pp60<sup>src</sup> Phosphorylates and Activates Low Molecular Weight Phosphotyrosine-protein Phosphatase*

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Stefania Rigacci†, Donatella Degl’Innocenti, Monica Bucciantini, Paolo Cirri, Andrea Berti‡, and Giampietro Ramponi

From the Department of Biochemical Sciences, University of Firenze, Viale Morgagni 50, 50134 Firenze, Italy

Low M<sub>v</sub>, phosphotyrosine-protein phosphatase belongs to the non-receptor cytosolic phosphotyrosine-protein phosphatase subfamily. It has been demonstrated that this enzyme dephosphorylates receptor tyrosine kinases, namely the epidermal growth factor receptor in vitro and the platelet-derived growth factor receptor in vivo.

Low M<sub>v</sub>, phosphotyrosine-protein phosphatase is constitutively tyrosine-phosphorylated in NIH/3T3 cells transformed by pp60<sup>src</sup>. The same tyrosine kinase, previously immunoprecipitated, phosphorylates this enzyme in vitro as well. Phosphorylation is enhanced using phosphatase inhibitors and phenylarsine oxide inactivated phosphatase, consistently with the existence of an auto-dephosphorylation process. Intermolecular dephosphorylation is demonstrated adding the active enzyme in a solution containing the inactivated and previously phosphorylated one. This tyrosine phosphorylation correlates with an increase in catalytic activity. Our results provide evidence of a physiological mechanism of low M<sub>v</sub>, phosphotyrosine-protein phosphatase activity regulation.

Low M<sub>v</sub>, phosphotyrosine-protein phosphatase (PTPase)<sup>1</sup> is an enzyme that possesses the characteristic PTPase CXXXXXR motif in the active site (1). The cysteine residue present in this sequence is absolutely necessary for the activity of the enzyme, as it forms a phosphohemideterminate during the reaction mechanism (1, 2). The phosphate-binding loop shows a striking structural similarity to the same loop of the cytosolic phosphotyrosine-protein phosphatase PTP1B. All these features, together with the overall recently described α/β structure (3), identify low M<sub>v</sub>, PTPase as belonging to the PTPase family. The enzyme was originally localized in the cytosol (4), and it can be considered a non-receptor cytosolic PTPase. Its structure does not display any features that can mediate its stable or temporary association with the membrane as occurs in the case of a PTPase containing Src-homology 2 (SH2) domains (SHP, SH-PTP1), myristoylation sites (a PTPase from Dictyostelium yeast), or cytoskeletal protein-like domains (a PTPase found in Helicobacter cells) (5). Nevertheless, its ability to act upon membrane substrates has been demonstrated: it dephosphorylates the EGF-r in vitro (6) and particularly the PDGF-r in vivo (7). When the enzyme is overexpressed in the NIH/3T3 cell line, the response to PDGF is inhibited dramatically, and the receptor appears to be phosphorylated to a much lesser degree. This evidence suggests that the PDGF-activated receptor can be down-regulated by low M<sub>v</sub>, PTPase. This was further confirmed by experiments performed in NIH/3T3 cells transfected with the negative dominant form of the enzyme, obtained by substituting the Cys-12 residue with Ser, showing the co-immunoprecipitation of PDGF-r with low M<sub>v</sub>, PTPase.<sup>2</sup>

Little is known about the regulation of the activity of cytosolic PTPases. From some research it appears that these enzymes can be subject to tyrosine phosphorylation, and, for some of them, the regulatory meaning of such modification has been demonstrated. PTP1C, containing two SH2 domains, is phosphorylated in vivo in response to colony stimulating factor 1 (CSF-1) (8) and insulin stimulation (9) and following v-src transformation of fibroblasts (10). It is also tyrosine-phosphorylated by pp60<sup>src</sup> in vitro, with a resulting increase in its activity. Another PTPase that is highly homologous to the preceding, named PTPID or Syp, is tyrosine-phosphorylated in response to EGF or PDGF stimulation (11) and in cells transformed by the tyrosine kinase pp120<sup>src-abl</sup> (12). In this case, it has been hypothesized that the modification may mediate the enzyme association with the SH2 domains of other tyrosine-phosphorylated proteins, which may become substrates of the PTPase activity.

pp60<sup>src</sup> is a widely expressed tyrosine kinase that can associate with membranes thanks to an amino-terminal acylation motif. This kinase is implicated in various cell functions, including cell to cell contact, neural differentiation, and cell proliferation (13), being known to be located downstream of the PDGF receptor in the mitogenic signal cascade that proceeds from this growth factor stimulation (14). It is known to phosphorylate a number of substrates, among which we can cite GTPase activating protein of Ras (GAP), pp125 focal adhesion kinase (FAK), pp120, and other components of the cytoskeleton (15, 16). Furthermore, it can autophosphorylate on Tyr-416 (13).

The oncogenic form, pp60<sup>src</sup>, is permanently active since it lacks the carboxyl-terminal region comprising Tyr-527 (17). In fact, the phosphorylation in pp60<sup>src</sup> of this residue determines its intramolecular binding with the SH2 domain, resulting in the inaccessibility of the active site with the subsequent inhibition of the kinase activity (18).

In this study we demonstrate that low M<sub>v</sub>, PTPase is phosphorylated by Src-kinase in vitro and in NIH/3T3 cells transformed by v-src. This in vitro phosphorylation causes an in-

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† To whom correspondence should be addressed. Tel.: 39-55-413-765; Fax: 39-55-422-2725.

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1 The abbreviations used are: PTPase, phosphotyrosine phosphatase; EGF, epidermal growth factor; EGF-r, epidermal growth factor receptor; PDGF, platelet-derived growth factor; PDGF-r, platelet-derived growth factor receptor; Pao, phenylarsine oxide; SH2, Src-homology 2; CSF-1, colony stimulating factor 1; pNPP, p-nitrophenyl phosphate; PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
increase in the enzyme activity and could be a way to regulate it in vivo as well.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—v-src transformed NIH/3T3 murine fibroblasts were obtained from the Laboratory of Cellular and Molecular Biology of the NCI, National Institutes of Health and have been described (19, 20). The cells were routinely cultured in a humidified atmosphere of 5% CO₂ at 37 °C in Dulbecco’s modified Eagle’s medium (Sigma D7777) supplemented with 10% fetal calf serum (complete medium). The cells were co-transfected with pSVPTP plasmid, containing a synthetic DNA sequence coding for low Mr, PTase (21) under the control of the SV40 early region promoter, and with pK0neo, expressing neomycin (G418) resistance, using the calcium phosphate precipitation method as described previously (22). Stable G418-resistant clones were selected by supplementing the culture medium with 400 μg/ml antibiotics. The clones were periodically cultured in the same selective medium to maintain a stable expression.

Evaluation of PTase Overexpression—Northern blot analysis of total RNA was performed under standard conditions, according to Ramponi et al. (22), using the 32P-labeled PTase synthetic gene as a probe. The low M₀ PTase content was evaluated by a noncompetitive immunoenzymatic assay as described previously (23). The activity of the overexpressed enzyme was checked in both parental and low Mr PTase transfected cell lysates using p-nitrophenylphosphate (pNPP) as a substrate. The overexpressed activity was totally inhibited by vanadate.

Electrophoresis and Immunoblotting—The cells were lysed in 66 mM Tris-HCl, pH 6.8, containing 2% SDS, 10 mM EDTA, 10% glycerol, and 0.1 mM sodium vanadate and centrifuged at 13,000 rpm, the total protein content was determined by the biocinchonic acid solution (BCA) protein reagent assay; equal protein amounts from whole cell extracts were subjected to SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and then electrotransferred to a nitrocellulose membrane (Sartorius). After blocking with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) (25 mM Tris, pH 7.4, 0.8% NaCl, 0.2% KCl) containing 0.1% Tween 20 for 1 h, the membrane was incubated with RC20 anti-phosphotyrosine antibody conjugated to horseradish peroxidase (Transduction Laboratories) for an additional 60 min. After an extensive washing with TBS-T (0.5% Tween 20 in TBS), the immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) (Amer sham) detection following the manufacturer’s instructions. After incubation for 30 min at 50 °C in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) and extensive washing, the membranes were reprobed with polyclonal anti-low Mr, PTase horseradish peroxidase-conjugated antibodies (produced and conjugated in our department as described previously (23)), following the same procedure.

pNPP Immuno precipitation—v-src transformed NIH/3T3 cells were grown to confluence on a 100-mm dish, washed twice with phosphate-buffered saline, and then collected in 500 μl of IP buffer (10 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate). After 10 min on ice, the cell lysate was centrifuged at 12,000 rpm for 10 min, 10 μl of mouse anti-pNPP (20) monoclonal antibody (Chemicon International Inc. MAB4030, ascites (fluid)) were added to the supernatant that was maintained on ice for 1 h. 25 μl of Protein A-Sepharose (Sigma), previously incubated for 1 h on ice with 5 μl of rabbit antibody to mouse IgG, were mixed with the above lysate. After 1 h of incubation in a shaker at 4 °C, the beads were collected by centrifugation and washed four times with 1 ml of IP buffer.

Expression and Purification of Recombinant Low Mr, PTase—The enzyme used in the in vitro phosphorylation experiments was obtained as a fusion protein with glutathione S-transferase (24). The fusion protein was cleaved by thrombin, and PTase was then purified. The enzyme was fully active.

Low Mr, PTase Inactivation—The recombinant enzyme was incubated in 30 min on ice in TBS containing 1 mg/ml BSA and 100 μM PAO to achieve its total inactivation. Unreacted PAO was removed by dialysis.

In Vitro Low Mr, PTase Phosphorylation and Dephosphorylation—To evaluate the extent of PTase phosphorylation, the kinase reaction mixture included PAO-inactivated recombinant low Mr, PTase (1 μg), kinase buffer (25 mM Hepes pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, 0.1% Nonidet P-40, 100 mM NaCl, 10% glycerol), BSA (1.5 μg), 100 μM sodium vanadate, 0.2 mM ATP, 22.5 μCi of [γ-32P]ATP, and Protein A-Sepharose beads (5 μl) bound to pp60 V-src, in a total reaction volume of 15 μl. The control reaction mixture did not include low Mr, PTase. The reaction was carried out at 30 °C. Samples were withdrawn at 30 and 120 min, mixed with 2 × Laemmli sample buffer, boiled, and run on a 12% SDS-PAGE. The gel was then dried and exposed to a film (Kodak x-ray) to obtain the autoradiography.

To follow the auto-dephosphorylation of the enzyme, 1 μg of inactive low Mr, PTase, which had been previously v-Src-phosphorylated, was mixed with 0.05 μg of active recombinant PTase. The incubation was carried out in 10 mM acetate, pH 4.9, 1 mM EDTA, 1 mM dithiothreitol at room temperature. The control reaction mixture did not include the active PTase. At various time intervals, aliquots of the reaction mixture were sampled and boiled in 2 × Laemmli sample buffer. After SDS-PAGE, the PTase phosphorylation extent was evaluated by autoradiography.

To subsequently determine the activity of the phosphorylated enzyme, the kinase reaction included active low Mr, PTase, kinase buffer containing 60 μM 2-mercaptoethanol, BSA, γ-thioATP (Boehringer Mannheim), and Protein A-Sepharose beads bound to pp60 V-src in the same quantities as in the phosphorylation test. The control reaction mixture did not include γ-thioATP. The incubation was carried out at 30 °C. Supernatant aliquots were assayed at various time intervals for their PTase activity.

PTase Activity Assay—The activity of the enzyme was determined at 37 °C in 0.1 mM acetate, 1 mM EDTA, pH 5.5, using alternatively as substrates p-nitrophenylphosphate (pNPP) or the peptide corresponding to the sequence 767–776 of the PDGF receptor (0.5 mM), containing a phosphotyrosine.

When the phosphopeptide was the substrate, the reaction was stopped by the addition of trichloroacetic acid (3% final concentration), and the mixture was centrifuged at 18,000 rpm for 5 min. Aliquots of the supernatant were used to determine the phosphate released by the PTase activity with the malachite green colorimetric assay (25).

Determination of Growth Kinetics—Control and low Mr, PTase overexpressing v-src-NIH/3T3 cells were seeded at low density (10,000 cells/cm²) on a multiwell plate in complete medium. Cell number was determined every 24 h for the following 3 days using a Bürker chamber. Four wells were counted each day.

Phosphoamino Acid Analysis—An aliquot of the in vitro phosphorylated low Mr, PTase was sampled from the supernatant of the reaction mixture before Laemmli buffer addition and was partially digested with 6 M HCl at 110 °C for 2 h. The phosphoamino acid separation was performed in high pressure liquid chromatography on a DEAE-column equilibrated with 10 mM potassium phosphate, pH 3.0, 12.5% methanol. After a post-column derivatization with o-phthalaldehyde, the amino acids were detected through their fluorescence emission and collected.

The 32P-labeled amino acids were quantified in a liquid scintillation counter.

RESULTS AND DISCUSSION

After transfection with pSVPTP, v-src transformed NIH/3T3 cells displayed high overexpression levels of low Mr, PTase as demonstrated by its determination in a noncompetitive immunoenzymatic assay and confirmed by Northern blot RNA analysis. The overexpressed enzyme was active on pNPP, and this activity was completely sensitive to vanadate inhibition, as all PTases are known to be.

To investigate the possible effects of such PTase activity on the cellular phosphorylation balance, we obtained whole cell lysates and analyzed them by SDS-polycrylamide gel electrophoresis, followed by immunoblotting with anti-Tyr(P) antibody (Fig. 1A). No significant differences between PTase overexpressing v-src transformed NIH/3T3 and control cells were evidenced, except for a protein of approximately 18 kDa that reacted significantly with anti-Tyr(P) antibodies in cells overexpressing the low Mr, PTase. The nitrocellulose membrane was then stripped and incubated with anti-PTase horseradish peroxidase-conjugated antibodies (Fig. 1B). The ECL exposition revealed that the tyrosine-phosphorylated 18-kDa protein in PTase-overexpressing cells was specifically recognized by anti-low Mr, PTase antibodies.

Similar analysis conducted on PTase-transfected NIH/3T3 cells, either normal or transformed by the deregulated tyrosine kinase coded by v-erbB, did not show any tyrosine phosphorylation on the overexpressed enzyme (data not shown). This
observation and the obvious evidence that Src tyrosine kinase activity is the major cause for Tyr phosphorylation in v-src transformed NIH/3T3 cell line, led us to hypothesize the direct involvement of this kinase in low Mr PTPase phosphorylation.

To verify this hypothesis we immunoprecipitated the pp60^src from v-src NIH/3T3 and used the immobilized enzyme in a kinase assay including low Mr PTPase and [γ-32P]ATP. In these conditions, the enzyme was poorly phosphorylated. The phosphorylation level increased notably if the PTPase was inactivated previously with PAO and vanadate was added to the kinase mixture, indicating a probable auto-dephosphorylation when the enzyme was active. The phosphorylation was evidenced by analysis of the reaction mixture on SDS-PAGE and autoradiography (Fig. 2).

The auto-dephosphorylation was confirmed by incubating PAO-inactivated and previously phosphorylated PTPase with the active enzyme. Fig. 3 shows that the enzyme was capable of dephosphorylating itself intermolecularly. Analysis of the phosphorylated amino acids conducted on the in vitro phosphorylated enzyme revealed the significant presence of phosphotyrosine, while neither phosphoserine nor phosphothreonine was detected.

Other PTPases are known to be substrates of tyrosine kinases, and, in some cases, the regulatory significance of the subsequent modification has been demonstrated: PTP1D-Syp is tyrosine-phosphorylated in response to the stimulation of receptors by various growth factors (11) as well as in cells transformed by the tyrosine kinase pp120^src-abl (12). In the former case, the authors report a slight activation of the enzyme, while in the latter case the authors did not find any variation in the enzyme activity, since it rapidly auto-dephosphorylates; in observing the co-immunoprecipitation of this PTPase with other tyrosine-phosphorylated proteins containing SH2 domains, they hypothesize that it may associate with them after its phosphorylation and catalyze their dephosphorylation. Another PTPase, named PTP1C, is phosphorylated in vivo in response to CSF-1 (8) and by the activated insulin receptor both in vivo and in vitro (9); evidence also suggests it is phosphorylated by pp60^src both in vivo and in vitro (10); in any case, a 4-fold activation follows this modification. The authors hypothesize a negative regulatory role of activated PTP1C on insulin signaling.

In order to assess if tyrosine phosphorylation of low Mr, PTPase could modulate its activity, we had to obtain a phosphorylated form of the enzyme in the absence of any PTPase inhibitor. For this reason, the kinase reaction, including immunoprecipitated pp60^src and PTPase, was carried out using γ-thioATP in place of ATP. The resulting thiophosphorylated residues were relatively resistant to phosphotyrosine phosphatase activity (26). The activity of the thiophosphorylated enzyme was assayed at various times during the kinase reaction using both pNPP and a tyrosine-phosphorylated peptide of the PDGF receptor as substrates. We observed an activation that proceeded along with the phosphorylation of the enzyme. As can be seen in Fig. 4, the activity of the phosphorylated enzyme reaches 190% on pNPP and 140% on the phosphopeptide, given the activity of the control mixture containing the unphosphorylated enzyme as 100%.

We had previously observed that parental NIH/3T3 cells overexpressing low Mr, PTPase showed a reduced mitogenic potential, and this effect was particularly striking when cells were stimulated with PDGF-BB. We demonstrated that such an effect was associated with the ability of the overexpressed PTPase to dephosphorylate the activated PDGF-r, thus suppressing the mitogenic signal which departs from the receptor after its binding with the specific growth factor (7). The co-immunoprecipitation of the PDGF-r with the dominant negative mutant of the PTPase in cells overexpressing this form of the enzyme confirms this result.2

The overexpression of low Mr, PTPase in NIH/3T3 cells transformed by v-erbB, coding for the truncated form of the EGF receptor, led to an analogous reduction in growth rate and in the ability to form colonies in soft agar (22). In this case as well, we presumed a direct activity of the PTPase on the autophosphorylated product of v-erbB, this hypothesis being validated by the observed activity of low Mr, PTPase on the autophosphorylated EGF-r in vitro (6).

The preceding evidence points to a selective activity of this
PTPase on receptor tyrosine kinases. In order to ascertain a possible effect of this enzyme on the mitogenic signal cascade downstream of growth factor receptors, we decided to transfect the enzyme in v-src transformed NIH/3T3 cells. It is well known that pp60
src
is located downstream of the PDGF-r in the mitogenic signal cascade (14). The mitogenic signal that proceeds from pp60
src
seems to be independent of PDGF stimulation; in fact, if we stimulate PTPase overexpressing v-src NIH/3T3 fibroblasts with PDGF-BB, we do not observe any phosphorylation of the specific receptor or any increase in the mitogenic rate with respect to control (data not shown).

PTPase-transfected v-src-NIH/3T3 show levels of overexpression much higher then those obtained when normal NIH/3T3 were transfected; moreover, the enzyme is activated when it is tyrosine-phosphorylated by pp60
src
in vitro. Nevertheless, no differences in the tyrosine phosphorylation pattern can be observed, except for the phosphorylation of the overexpressed Low Mr PTPase. Furthermore, when we evaluate the growth rate of PTPase-transfected v-src-NIH/3T3, we do not find any significant difference as compared to control v-src fibroblasts (Fig. 5). This result could be an indirect confirmation of the fact that, at least in NIH/3T3 fibroblasts, the phosphorylated PDGF receptor is a target of low Mr PTPase. Furthermore, when we evaluate the dephosphorylation of the PDGF-r might be obtained. The low Mr PTPase could thus participate in a feedback control mechanism of PDGF receptor activity.

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