

## Modulation of Protein Tyrosine Phosphatase 1B by Erythropoietin in UT-7 Cell Line

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### Key Words

Erythropoietin • Protein Tyrosine Phosphatase 1B • UT-7 cell line

### Abstract

**Background/ Aims:** Since the reversible phosphorylation of tyrosyl residues is a critical event in cellular signaling pathways activated by erythropoietin (Epo), attention has been focused on protein tyrosine phosphatases (PTPs) and their coordinated action with protein tyrosine kinases. The prototypic member of the PTP family is PTP1B, a widely expressed non-receptor PTP located both in cytosol and intracellular membranes via its hydrophobic C-terminal targeting sequence. PTP1B has been implicated in the regulation of signaling pathways involving tyrosine phosphorylation induced by growth factors, cytokines, and hormones, such as the downregulation of erythropoietin and insulin receptors. However, little is known about which factor modulates the activity of this enzyme. **Methods:** The effect of Epo on PTP1B expression was studied in the UT-7 Epo-dependent cell line. PTP1B expression was analyzed under different conditions by Real-Time PCR and Western blot, while PTP1B phosphatase

activity was determined by a p-nitrophenylphosphate hydrolysis assay. **Results:** Epo rapidly induced an increased expression of PTP1B which was associated with higher PTP1B tyrosine phosphorylation and phosphatase activity. The action of Epo on PTP1B induction involved Janus Kinase 2 (JAK2) and Phosphatidylinositol-3 kinase (PI3K). **Conclusion:** The results allow us to suggest for the first time that, besides modulating Epo/Epo receptor signaling, PTP1B undergoes feedback regulation by Epo.

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### Introduction

The phosphorylation of tyrosyl residues in proteins is a critical event in many signaling pathways involved in proliferation, differentiation, and cellular response to extrinsic factors [1]. Since protein tyrosine phosphorylation is closely controlled by both protein tyrosine kinases (PTKs) and tyrosine phosphatases (PTPs), a disturbance of the balance between the activity of these enzymes leads to abnormal tyrosine phosphorylation which might be related to different human

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pathologies, such as cancer, diabetes, or inflammatory diseases [2-5].

The prototype of the PTPs superfamily is the widely expressed PTP1B. This enzyme exists as a 435 aminoacid form of 50 kDa [6], with an N-terminal catalytic domain (PTP domain) followed by two proline-rich motifs, and is located on the endoplasmic reticulum (ER) through a small hydrophobic stretch found in its C-terminus [7]. Several biochemical studies have implicated PTP1B in the attenuation of various PTKs signaling pathways, such as those related to the receptors of erythropoietin, insulin, insulin-like growth factor I, epidermal growth factor and platelet-derived growth factor [8-12].

The erythropoietin receptor (EpoR) is essential for the regulation of proliferation, differentiation, and survival of erythroid cells. Following ligand binding, the receptor conformation changes [13, 14], subsequently inducing phosphorylation and activation of the associated kinases. Tyrosine-phosphorylated EpoR then serves as a docking site for other signaling proteins, like JAK2 and PI3K, which mediate the cellular response to Epo. Even though termination of the Epo/EpoR activation pathway is mainly attributed to the cytosolic SH-PTP1 protein [15], it has recently been reported that PTP1B also dephosphorylates Epo-stimulated EpoR, thus participating in the downregulation cascade of Epo-mediated transduction [12].

The current study was performed to clarify possible interactions between PTP1B and activated EpoR intracellular pathway, using the UT-7 human leukemic cell line as an Epo-dependent cell model. The results let us postulate the existence of reciprocal regulation between Epo and PTP1B.

## Materials and Methods

### Materials

All chemicals used were of analytical grade. Bovine serum albumin (BSA), Ly294002, AG490, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium o-vanadate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin A were obtained from Sigma-Aldrich; Iscove's Modified Dulbecco's Medium (IMDM), Taq DNA polymerase, dNTPs, and specific primers for PTP1B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Invitrogen Life Technologies; polyclonal anti-PTP1B antibody (SC-14021, raised against aminoacids 301-435 mapping at the C-terminus of PTP1B) from Santa Cruz Biotechnology; monoclonal anti-PTP1B (PH-01, epitope: PTP1B active site) from Calbiochem; polyclonal anti-actin (A4700) from Sigma, monoclonal anti-phosphotyrosine (anti-PY) antibody (P-11120) and Protein A-

agarose from BD Transduction Laboratories; Trizol Reagent from Gibco BRL; nitrocellulose (NC) membranes (Hybond), chemiluminiscent system kit (ECL), and Ready To Go T-Primed First-Strand Kit from Amersham Bioscience; agarose from Promega; ethidium bromide from Mallinckrodt; Sybr Green I nucleic acid stain from Roche; sodium dodecylsulfate (SDS), acrylamide, bis-acrylamide, Triton X-100, Folin-Ciocalteu's reagent, Tween 20, and p-nitrophenyl phosphate (pNPP) from Merck; fetal bovine serum (FBS) (Bioser) and penicillin-streptomycin (PAA Laboratories) from GENSA and recombinant human erythropoietin (rhuEpo, Hemax) from Biosidus.

### Cell line and culture

UT-7 cell line was kindly provided by Dr. Patrick Mayeux (Cochin Hospital, Paris, France). Initially established from bone marrow cells obtained from a patient with acute megakarioblastic leukemia, this cell line shows complete growth dependence on Epo. Stock cultures were maintained in IMDM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 U/ml Epo, by replacement of the medium every 3-4 days [16]. Cell cultures were developed at 37°C, in an atmosphere containing 5% CO<sub>2</sub> and 100% humidity. Proliferation and cell viability were evaluated by the Trypan blue exclusion test.

### MTT Assay

The MTT reduction assay modified from that of Mosmann [17] was used to assess cell viability as a measure of Ly294002 or AG490 cytotoxicity. Briefly, after cells had been subjected to appropriate treatments in microplates, MTT was added to each well at a final concentration of 0.5 mg/ml and the cells were incubated for 2 h at 37°C. The medium was removed and the pellet washed with PBS. Finally 100 µL of 0.04 M HCl in isopropanol was added to solubilize the blue formazan product (reduced MTT) and the absorbance was read at a 570 nm test wavelength with a 690 nm reference filter (Microplate Reader BioRad, Model 680).

### Precipitation and Western Blotting

Cells were washed with ice-cold phosphate buffer saline (PBS) containing 1 mM sodium o-vanadate and lysed on ice-cold hypotonic lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100), with 1 mM PMSF, 1 µg/ml aprotinin, 2 µg/ml leupeptin, 1.37 µg/ml pepstatin A, in a ratio of 200 µl to 10<sup>7</sup> cells. After 30 min-incubation on ice, cell lysates were clarified by centrifugation for 15 min at 15,000 g at 4°C. Total proteins were quantified by the Lowry's method [18].

Monoclonal anti-PTP1B antibody (epitope: active site, Calbiochem) was added at 2 µg/ml final concentration and incubated at 4°C for 60 min with gentle agitation. Protein A-agarose was added and after an overnight incubation with rotation at 4°C, immunocomplexes were collected by centrifugation at 15,000 g for 15 min and washed twice with the lysis buffer.

Immunoprecipitates or cell lysates corresponding to 100 µg of total proteins were boiled for 3 min in the Laemmli sample buffer [19], fractionated on 12% polyacrilamide-SDS gel and electrophoretically transferred to nitrocellulose membrane during 1.5 h (transfer buffer: pH 8.3, 25 mM Tris, 195 mM glycine,

0.05% SDS, and 20% (v/v) methanol). The membranes were blocked by 1 h-incubation in TBS (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 and 0.5% skim-milk powder, and then incubated with appropriate concentrations of the specific antibody. After washing with TBS-0.1% Tween 20, the immunoblots were probed with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1,000) for 1 h at 20°C and washed. Specific antibody signals were detected using the enhanced chemiluminescence system, ECL kit, and the bands detected by using a Fujifilm Intelligent Dark Box II (Fuji) equipment coupled to a LAS-1000 digital camera.

Anti  $\beta$ -actin polyclonal antibody was also used in western detection as control for sample loading variations.

#### *Enzyme Activity Assay*

Aliquots of cell lysate equivalent to 1 mg total protein were immunoprecipitated with anti-PTP1B (epitope: Carboxyl terminus, Santa Cruz) as indicated and immunoprecipitates washed with PTP assay buffer (18.5 mM HEPES, pH 7.2, 7.5 mM NaCl, 1.85 mM EDTA, 5 mM DTT). The PTP activity was measured in a 200  $\mu$ l final volume by incubation at 37°C for 30 min in PTP assay buffer containing 2.4 mM pNPP. The product absorption was determined at 415 nm in a microplate reader. The correction for non-enzymatic hydrolysis of pNPP was evaluated by measuring the absorbance in the absence of enzyme.

#### *Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA was isolated by means of the Trizol Reagent, according to Chomczynski and Sacchi [20]. RNA concentration was estimated by measuring the optical density at 260 nm and then, 4.5  $\mu$ g RNA was reverse transcribed by using Ready-To-Go T-Primed First-Strand Kit. An aliquot (1  $\mu$ l) of a dilution of cDNA solution (1:10) was amplified by PCR employing specific primers for PTP1B (sense: 5'-GGGGTGTGTCATGCTCAA-3' and antisense: 5'-GCCATGTGGTATAGTGGAAATGT-3') and for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [21]. PCR reactions were performed in a Mastercycler Gradient (Eppendorf) at the following PCR cycling conditions: an initial denaturing step at 94°C for 5 min, 28 cycles repeating 94°C for 45 s, 59.5°C for 45 s and 72°C for 45 s and a final elongation step at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide. Gels were photographed and analyzed with the ImageGauge software.

#### *Quantitative Real-Time PCR*

Real-Time PCR for PTP1B was performed in a volume of 25  $\mu$ l of a mixture containing cDNA (1:10 dilution), 0.20 mM dNTPs, specific primers at 0.25  $\mu$ M (see Material and Methods), 3 mM MgCl<sub>2</sub>, 2 U Taq DNA polymerase and 1:30,000 SYBR Green Stain. Real-Time PCR reactions were performed in a DNA Engine Opticon (MJ Research Inc.) and the amplification program consisted of an initial denaturing step (94°C for 5 min), followed by 40 cycles (each of 94°C for 45 s, 59.5°C for 45 s and 72°C for 45 s). Sample quantification was normalized to endogenous GAPDH which was also quantified by Real-Time PCR following

the same protocol as that for PTP1B. Acquisition of the fluorescence signal from the samples was carried out at the end of the elongation step. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA obtained from UT-7 cells maintained with IMDM 10% FBS and 1U/ml Epo. All samples were run in duplicate and the experiment was repeated three times with independently isolated RNA.

#### *Statistics*

Results are expressed as mean  $\pm$  standard error (S.E.M). Comparison among groups was carried out by the Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U-test when corresponding. Least significant difference with  $P < 0.05$  was considered as the criterion for statistical significance.

## **Results**

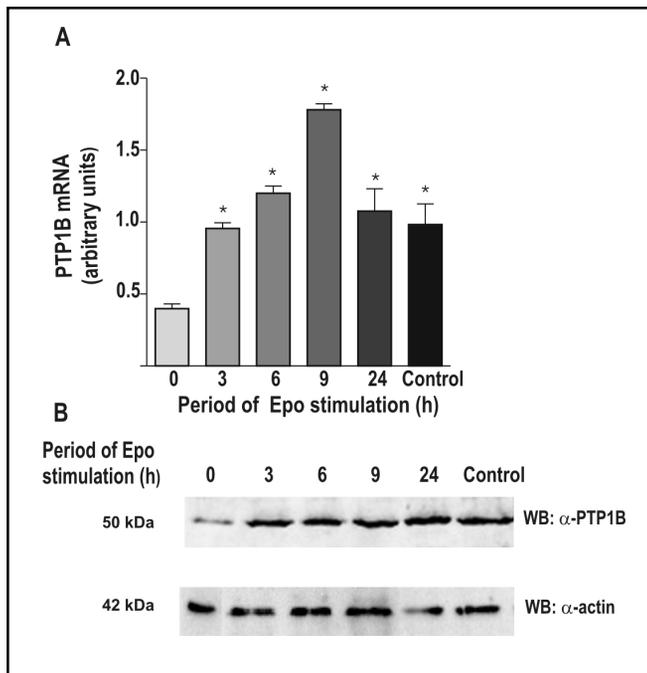
### *Erythropoietin induces the expression and activity of PTP1B*

Previous reports have proved that PTP1B participates in the downregulation of Epo-mediated signal transduction through dephosphorylation of Epo-stimulated EpoR [12]. To assess the influence of Epo in the modulation of PTP1B expression, we first investigated possible changes in the expression level of PTP1B after UT-7 cells had been deprived and subsequently stimulated by Epo. These cells, strongly dependent on Epo for growth and survival, were cultured in the absence of serum and Epo for 18 h and after this starvation period, they were stimulated with 10 U/ml Epo (without medium change or addition of other supplements) for different times.

Figure 1 shows the effect of Epo stimulation upon the PTP1B expression analyzed by Real-Time PCR (A) and Western Blotting (B). UT-7 cells cultured without serum and the erythropoietic growth factor ( $t = 0$ ) showed low PTP1B expression at both mRNA and protein levels, which was already increased by a 3 h-Epo stimulation period. From this point, protein expression level was almost constant whereas PTP1B mRNA reached its maximum by 9 h of Epo stimulation. Finally, PTP1B mRNA decreased to control levels (UT-7 cells maintained with 1U/ml Epo).

PTP1B phosphatase activity, measured by the rate of pNPP hydrolysis, was also increased by Epo stimulation during 3h. After this point, the enzymatic activity decreased to control levels (Fig. 2).

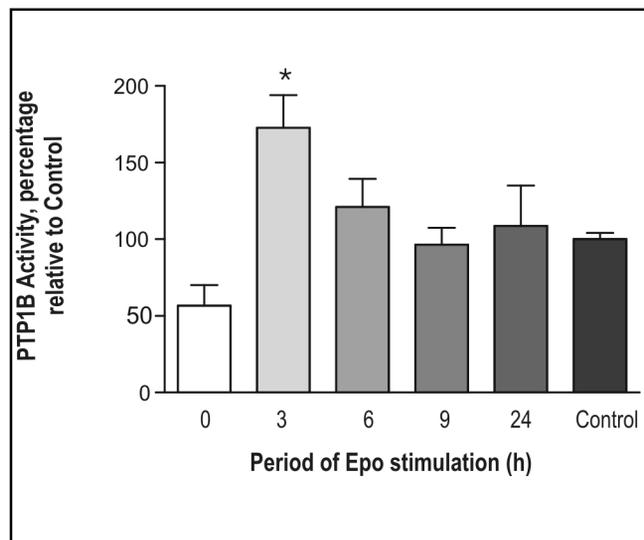
Regarding the percentage of viable cells, the Trypan blue exclusion test showed no significant differences among treatments, being the observed viability range between 59.5% and 68.8%, throughout the period tested.



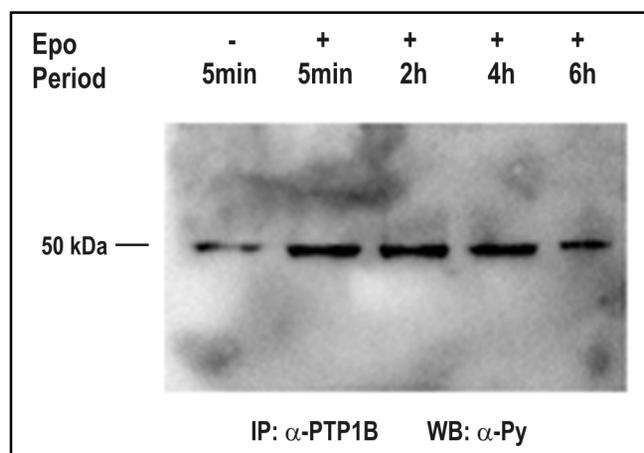
**Fig. 1.** Epo modulates PTP1B expression. Cells ( $1 \times 10^7$ ) were cultured in IMDM medium without serum and rhuEpo. After 18 h starvation ( $t = 0$  h) cells were stimulated with 10 U/ml Epo during different periods (3, 6, 9 or 24 h). Control cells were maintained with 1U/ml Epo during the whole assay. Total RNA was extracted and PTP1B expression quantified by Real-Time PCR (A). Cell lysates were also prepared and resolved by SDS-PAGE (B). The proteins were transferred to NC membranes and detected by immunoblotting using anti-PTP1B antibody. Each bar represents mean value  $\pm$  standard error ( $n = 3$ ). Figure 1 shows representative patterns of 3 separate experiments. (\*) Significant differences with respect to  $t = 0$  h ( $P < 0.01$ ).

#### *Erythropoietin induces PTP1B phosphorylation*

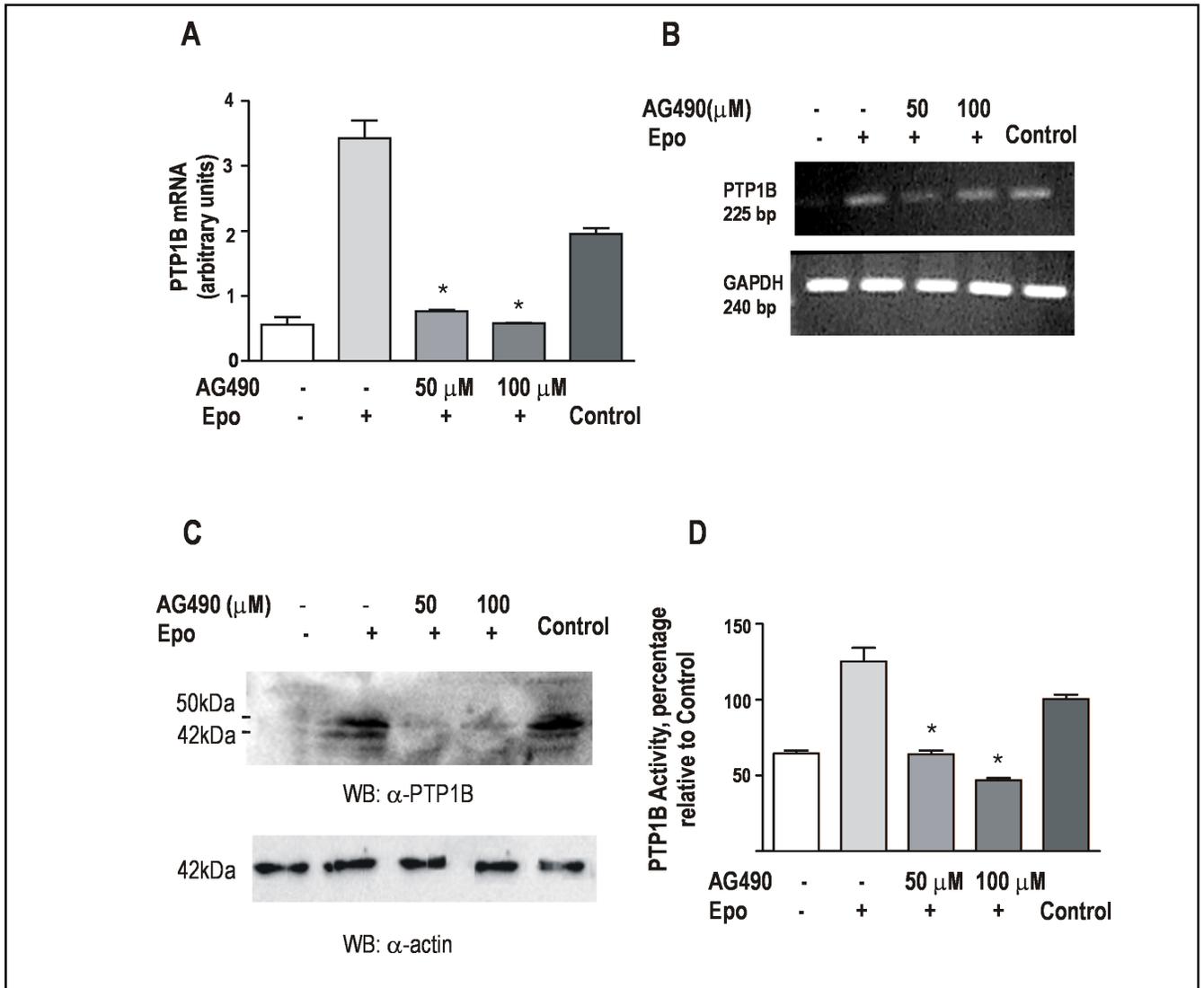
Since it is known that both epidermal growth factor and insulin stimulate PTP tyrosine phosphorylation which increases its phosphatase activity [22], it was interesting to investigate if Epo could also modulate PTP1B tyrosine phosphorylation. UT-7 cells were starved of serum and Epo for 18 h and then stimulated with 10 U/ml Epo (without medium change or addition of other supplements) for short periods. Afterwards, cell viability was evaluated and cell lysates were immunoprecipitated with anti-PTP1B antibody followed by Western blot analysis with anti-PY antibody. Figure 3 shows the expected kinetic pattern of a transient PTP1B tyrosine phosphorylation induced by Epo, which was already detectable by 5 min after Epo stimulation and declined within 6 h while cell viability was not significantly altered during the whole period.



**Fig. 2.** Epo modulates PTP1B activity. Cells ( $1 \times 10^7$ ) were treated as described in figure 1. Total lysates were prepared and PTP1B was immunoprecipitated with anti-PTP1B antibody. PTP1B phosphatase activity was determined by measuring the optical density (415 nm) of the product from the pNPP hydrolysis. Results are reported as the percentage activity with respect to that of control cells. Each bar represents mean value  $\pm$  standard error ( $n = 3$ ). (\*) Significant differences with respect to PTP1B activity shown at  $t = 0$  ( $P < 0.05$ ).



**Fig. 3.** Epo induces PTP1B tyrosine phosphorylation. Cells ( $1 \times 10^7$ ) were deprived of serum and Epo during 18 h. After the starvation period, cells were stimulated with 10 U/ml Epo (+) during 5 min, 2, 4 or 6 h, followed by cell lysis. Cells without Epo (-) were incubated with PBS for 5 min. Whole cell extracts were immunoprecipitated with anti-PTP1B ( $\alpha$ -PTP1B) and analyzed by Western blot probed with anti-phosphotyrosine antibody ( $\alpha$ -PY). The figure is representative of 3 separate experiments.



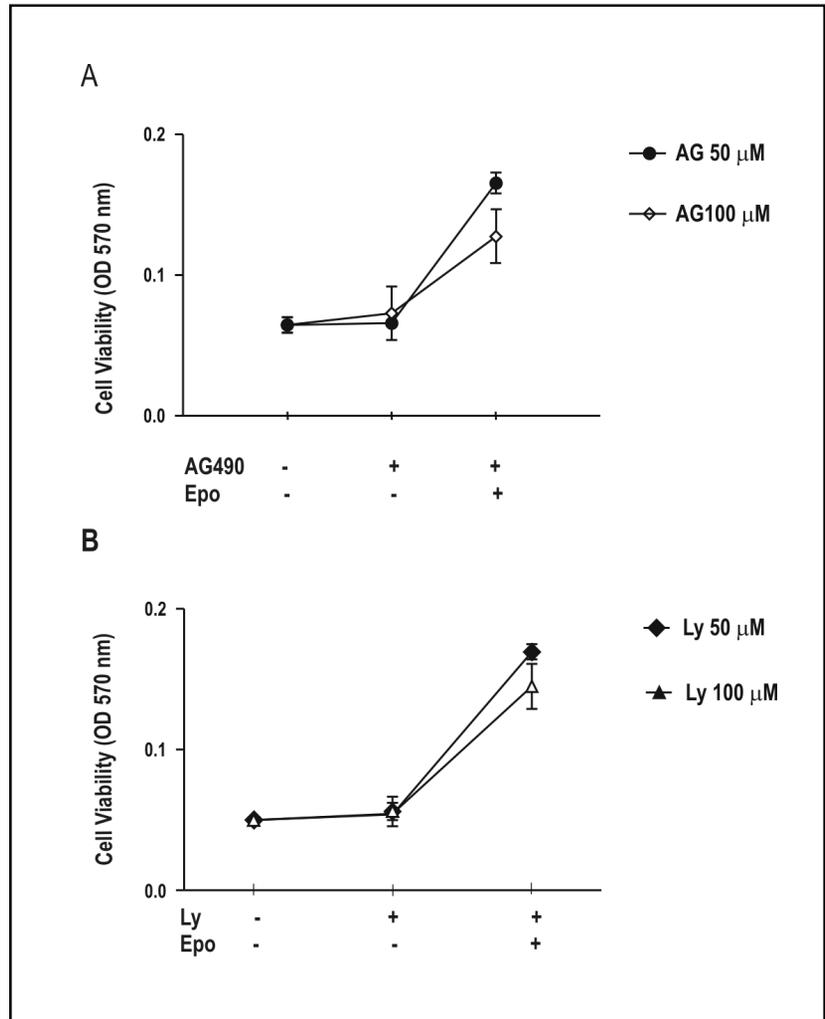
**Fig. 4.** JAK2 is implicated in PTP1B modulation by Epo. After 18 h of Epo deprivation, UT-7 cells were incubated in the presence of the indicated concentrations of the JAK 2 inhibitor AG490 for 5 h and subsequently stimulated with Epo for 3 h. Control cells were maintained with 1U/ml Epo during the whole assay. Then, cDNA and total cell lysates were prepared for quantitative Real-Time PCR (A) or qualitative RT-PCR (B) and Western blot analysis with anti-PTP1B antibody (C), respectively. Besides, an aliquot of total lysates was immunoprecipitated with anti-PTP1B to determine PTP1B activity (D) by measuring the optical density (415 nm) of the product from pNPP hydrolysis and reported as percentage activity relative to that of control cells. Each bar represents mean value  $\pm$  standard error ( $n = 3$ ). Figure 4 shows representative patterns of 3 separate experiments. (\*) Significant differences relative to the treatments with Epo and without inhibitor ( $P < 0.05$ ).

#### *JAK2 and PI3K are involved in PTP1B modulation by Epo*

To gain insight into the role of JAK2 and PI3K signaling in the Epo-induced PTP1B expression, assays were carried out in the presence of AG490 and Ly294002 (Ly), known as specific JAK2 and PI3K inhibitors, respectively. After 18 h of Epo deprivation, UT-7 cells were incubated for 5 h in the presence of two different

concentrations of AG490 or for 1 h with different concentrations of Ly. Then, the cells were stimulated with 10 U/ml Epo during 3 h (without medium change or addition of other supplements in any assay). Then, total cell lysates and cDNA were prepared for Western blot analysis with anti-PTP1B immunoreaction and Real-Time PCR, respectively. The MTT assay was used to detect cytotoxic effects.

**Fig. 5.** Incubation with AG490 or Ly does not affect cell viability. After 18 h of Epo deprivation, UT-7 cells were incubated in the presence of the indicated concentrations of AG490 for 5 h (A) or Ly for 1 h (B) and subsequently stimulated with Epo for 3 h. Cell viability was determined by MTT reduction analysis measured by optical density at 570 nm and a 690 nm reference wavelength. Each point represents mean value  $\pm$  standard error ( $n = 3$ ).



Cells treated with either 50 or 100  $\mu$ M AG490 showed decreased PTP1B mRNA and protein levels. Moreover, they were almost undetectable even in the presence of the lowest dose of the JAK2 inhibitor assayed (Fig. 4A, B and C). Similar modulation was observed upon PTP1B enzyme activity (Fig. 4D). Regarding cell viability, the preincubation with AG490 before Epo stimulation had no detectable effect (Fig. 5A).

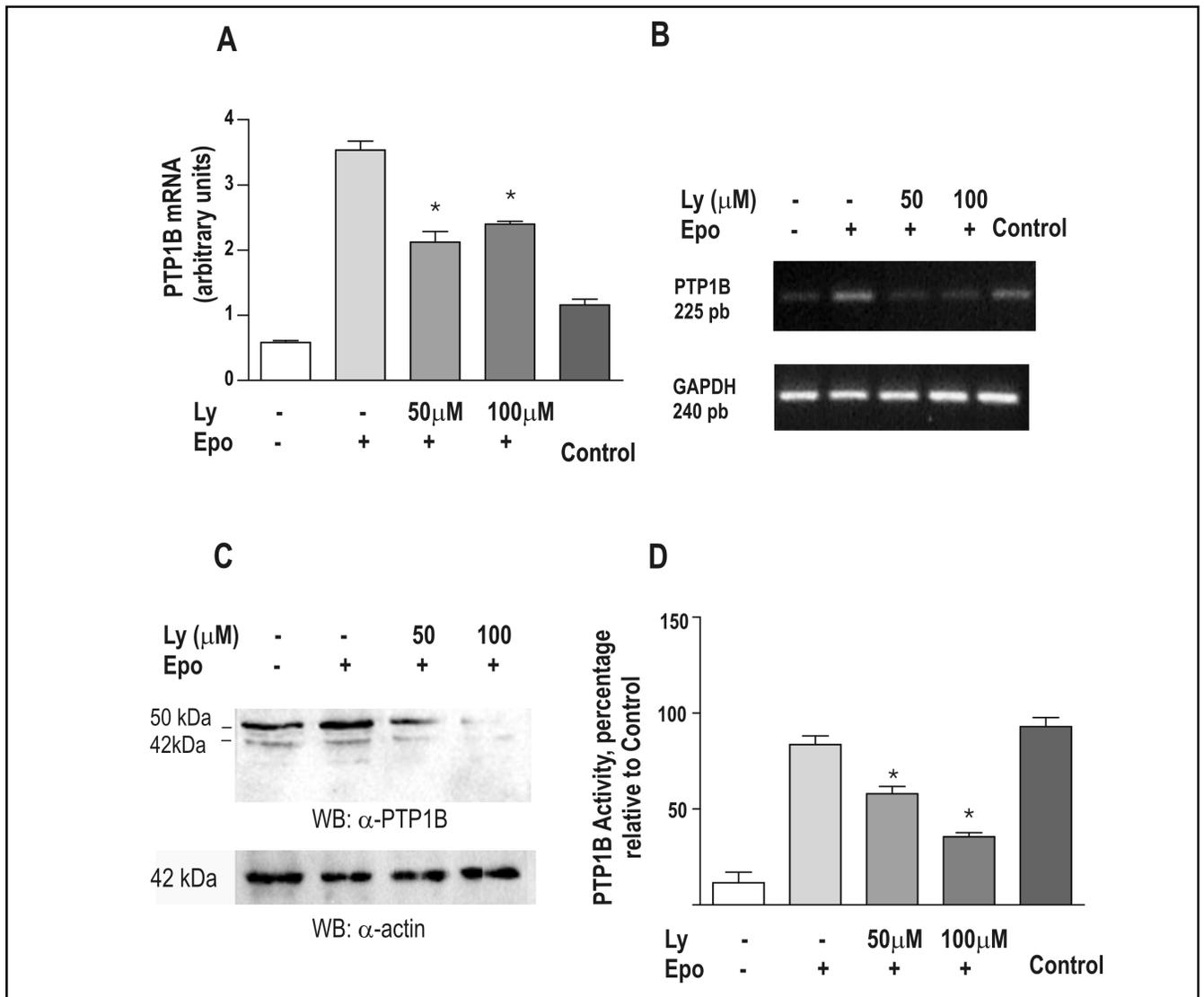
As shown in Figure 5B, MTT reduction was not suppressed after the incubation with Ly during 1 h, and it was even increased after Ly pretreatment followed by 3 h-stimulation with Epo. On the other hand, PTP1B expression was negatively regulated by Ly at mRNA and protein levels in different ways: while PTP1B mRNA downregulation did not depend on Ly dose and was not strong enough to decrease mRNA up to the initial level (Fig. 6A and 6B), the decrease in PTP1B protein level was evident in a Ly dose dependent manner (Fig. 6C). Moreover, the level of the enzyme detected in the presence of Ly was even lower than the one detected

after 18 h of Epo deprivation. In addition, the *in vitro* phosphatase activity underwent a similar dose-dependent modulation (Fig. 6D).

## Discussion

The phosphorylation of protein tyrosyl residues is a key element of the signaling pathways induced by different stimuli that regulate cellular responses such as growth, differentiation proliferation, and metabolism. Due to the requirement of a strictly controlled process, coordinated actions of protein kinases and phosphatases should be balanced.

Since phosphorylation is the activating event for the EpoR signaling, dephosphorylation is necessary to downregulate this activity. The distal end of the EpoR seems to act as a negative regulatory domain in which phosphorylated tyrosine residues are the docking sites for the tyrosine protein phosphatases that attenuates Epo



**Fig. 6.** Inhibition of PI3-kinase causes down-regulation of PTP1B expression. After 18 h of Epo deprivation, UT-7 cells were incubated in the presence of the indicated concentrations of Ly for 1 h and subsequently stimulated with Epo for 3 h. Control cells were maintained with 1 U/ml Epo during the whole assay. Then, cDNA and total cell lysates were prepared for quantitative Real-Time PCR (A) or qualitative RT-PCR (B) and Western blot analysis with anti PTP1B antibody (C), respectively. Besides, an aliquot of total lysate was immunoprecipitated with anti-PTP1B antibody to determine PTP1B activity (D) by measuring the optical density (415 nm) from pNPP hydrolysis. Results are expressed as percentage relative to that of control cells. Each bar represents mean value  $\pm$  standard error ( $n = 3$ ). (\*) Significant differences with respect to the assay with Epo and without inhibitor ( $P < 0.05$ ).

signaling [23]. Although much has been investigated about mechanisms mediated by PTKs in the intracellular pathways activated by the binding of Epo to its specific receptor, little is known about the role of individual PTPs in the regulation of this activity.

The target of this research was the enzyme PTP1B, the prototypic member of the PTP family. Since its discovery [24], continued investigation has revealed important data about PTP1B structure, regulation, and

function. In particular, the ability of this enzyme to antagonize insulin receptor signaling has been pointed out [10]. The expression of PTP1B is widely modulated in response to circumstances ranging from metabolic stress to cellular transformation [25, 26]. Some reports showed increased expression and/or activity of PTP1B in insulin-resistant states and obesity [27], especially in skeletal muscle and adipose tissue [28].

Recently, the participation of PTP1B in the

dephosphorylation of EpoR has also been reported. In vitro coexpression studies have shown that PTP1B can dephosphorylate Epo-stimulated EpoR, thereby downregulating the cascade of Epo-mediated signal transduction [12]. Nevertheless, no information has been reported about modulation of the PTP1B expression by this growth factor. Based on this notion, the present study has been designed to characterize the interaction between PTP1B and Epo/EpoR activated intracellular pathways in Epo-dependent cells, as well as to investigate whether PTP1B expression is modulated by the growth factor.

The model used was the UT-7 cell line, which is an Epo-responsive cell line co-expressing EpoR and PTP1B.

We found that Epo induced PTP1B expression which is associated to increased PTP1B tyrosine phosphorylation and enzyme activity. These conclusions came from different experiments that showed an increase in the phosphatase expression at both mRNA and protein levels after cell stimulation by Epo. The hormone upregulated PTP1B mRNA expression as soon as 3 hours, increased level that peaked at 9 hours and remained elevated for 24 hours. We then confirmed the rapid and sustained Epo-induced upregulation of PTP1B protein by Western blot analysis (Fig. 1B).

These findings agreed with a parallel increase in the activity of PTP1B. When this activity was measured by the hydrolysis of p-nitrophenylphosphate, the observed value before Epo activation (at  $t=0$ ) was not as low as expected (Fig. 2), presumably due to the existence of additional phosphatases precipitated by the polyclonal antibody.

Different previous studies have shown that PTP1B itself is a phosphoprotein. Bandyopadhyay et al [29] have shown that insulin stimulates phosphorylation of PTP1B on three tyrosine residues, which increased its phosphatase activity [22]. This action creates a negative feedback loop to downregulate insulin signaling. The epidermal growth factor receptor also phosphorylated PTP1B on a motif flanking Tyr-66 and this phosphorylation increased catalytic activity by near three-fold [30]. The phosphoserine content of PTP1B was also regulated by insulin stimulation but this modulation was found to be associated to a decreased PTP1B activity, which created a positive feedback mechanism for insulin signaling [31]. During mitosis, PTP1B serine residues are phosphorylated without alteration of enzymatic activity [30]. Other protein kinases, such as PKA or PKC, phosphorylated serine residues of PTP1B [32] but their effects on the enzymatic activity are not entirely clear. Based on these results, it has been suggested that tyrosine and serine

phosphorylation might regulate PTP1B activity in a temporal fashion depending on the stimulus and/or growth factor involved. Our results agree with these previous reported observations concerning tyrosine phosphorylation. Like insulin and other growth factors, the Epo-induced PTP1B tyrosine phosphorylation was associated with an increase in phosphatase activity. Opposite results were found by Sharlow E. et al [33] in a mouse cell line in which Epo failed to modulate the expression and tyrosine phosphorylation of hematopoietic cell phosphatase (HCP), another phosphatase implicated in EpoR signaling downregulation.

Thus, our findings suggest an important and different role of PTP1B as modulator in the erythropoietic process given that Epo is the principal hormone that regulates the proliferation and differentiation of erythroid precursor cells.

We also investigated the participation of downstream mediators, such as JAK2 and PI3K in assays carried out in the presence of AG 490 and Ly294002, known as specific JAK2 and PI3K inhibitors, respectively. Blocking the kinase activity of JAK2 and PI3K by their specific inhibitors abolished the Epo-mediated increase of PTP1B expression and partially suppressed its enzyme activity (Fig. 4 and 6). However, a higher concentration of PI3K inhibitor than of JAK2 inhibitor was necessary to observe a complete PTP1B inhibition, presumably caused by the fact that JAK2 is upstream PI3K in the Epo signaling pathway. Thus, the AG490 and Ly294002 assays indicated that Epo-stimulated JAK2 and PI3K appear to be necessary for inducing PTP1B, even though different degree of participation was observed.

Altered expression of PTP1B has been found in a number of disease conditions associated with aberrant tyrosine phosphorylation [22, 24-26]. In this context, enhanced expression of PTP1B was detected in animal models of diabetes as well as in patients with diabetes and insulin resistance [34]. Since erythropoiesis is regulated by a strict equilibrium between activities of both protein tyrosine kinases and tyrosine phosphatases, an imbalance due to a defect in phosphatase activity may allow uncontrollable cell proliferation suggesting a possible etiology for polycythemia vera [35]. On the other hand, after approximately twenty years of therapy with recombinant human erythropoietin (rhuEpo), poor responses to the treatment are frequently observed [36]. A contributing factor to this effect may be the elevation of PTP activity. Since PTP1B is involved in EpoR signal attenuation, an approach to improving rhuEpo treatment could be focused on modulation of EpoR intracellular signal

transduction cascade. PTP1B-specific regulatory factors may be expected to enhance Epo sensitivity and effectively act in the treatment of anemia or rhuEpo resistance [37]. That is why an understanding of such control mechanisms will be essential in order to achieve adequately management of the involved factors that would provide opportunities for new therapeutic strategies.

To our knowledge, the current study suggests for the first time that as well as modulating Epo/EpoR signaling, PTP1B undergoes a feedback regulation by Epo in which JAK2 and PI3K are involved. Thus, these results emphasize the possibility that changes in the level of expression of PTP1B may be involved in several human diseases.

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