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Protein tyrosine phosphatase 1B (PTP1B) is involved in the defective erythropoietic function of carbamylated erythropoietin

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1. Introduction

It is now recognized that the carbamylation of erythropoietin (Epo) in lysine residues makes this protein unable to act as a growth factor for erythropoiesis, while it retains its ability to protect neuronal cells from damage. In accordance with other authors (Brines et al., 2004; Leist et al., 2004; Coleman et al., 2006; Fantacci et al., 2006), we demonstrated that Epo and cEpo exerted similar protective effects on neuroblastoma-derived SH-SY5Y cells induced to apoptosis by staurosporine or TNF-α, while cEpo was unable to support proliferation of the UT-7 and TF-1 cell lines, both capable of erythroid differentiation (Chamorro et al., 2013). Furthermore, in this previous paper we presented an advance on the study of the differential effects between Epo and cEpo on hematopoietic and non-hematopoietic cells, since we reported the inability of cEpo to completely activate cell proliferation signaling pathways.

It is well known that the main function of Epo is the regulation of erythropoiesis. However, expression of the erythropoietin receptor (EpoR) in non-hematopoietic tissues indicates that Epo is a pleiotropic growth factor.

EpoR belongs to the same family of receptors as the growth hormone, the granulocyte colony-stimulating factor, the granulocyte-macrophage colony-stimulating factor and some interleukins. In the erythropoietic process, Epo induces homodimerization of EpoR on the cell surface. This protein does not possess an intrinsic tyrosine-kinase activity and requires accessory factors such as Janus kinase 2 (Jak2) for cell-surface transport and downstream signaling. The receptor is activated when a single Epo molecule binds two EpoR molecules and effectively "cross-links" them. Epo binding induces cross-phosphorylation of EpoR and Jak2, thus initiating the activation of downstream signaling pathways such as that of the signal transducer and activator of transcription (STAT5), the extracellular signal-regulated kinase (ERK), and the phosphatidylinositol 3 (PI3) kinase/AKT (Mulcahy, 2001; Arcasoy, 2008). The Epo/EpoR system has been shown to signal through protein kinases, anti-apoptotic proteins and transcription factors. Following activation, negative regulators of EpoR downmodulate responses. One of these mechanisms leads to receptor internalization, ubiquitination and degradation by proteasome (Walrafen et al., 2005). Since the phosphorylation of protein tyrosyl residues is a critical event in many signaling pathways involved in proliferation, differentiation,

Abbreviations: βcR, beta common receptor; cEpo, carbamylated erythropoietin; Epo, erythropoietin; EpoR, erythropoietin receptor; FBS, fetal bovine serum; Jak2, Janus kinase 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; pNPP, *p*-nitrophenylphosphate; PTP1B, protein tyrosine phosphatase 1B; SEM, standard error of the mean.

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and cellular response to Epo, phosphatases become important factors in the downregulation of such activities. Among these enzymes, the protein tyrosine phosphatase (PTP) family and its prototype PTP1B in particular, have been characterized in the attenuation of Epo signaling (Myers et al., 2001; Callero et al., 2007, 2011).

With respect to neuroprotection, cEpo appears to induce anti-apoptotic signaling pathways similar to those stimulated by Epo (Xu et al., 2009; Chamorro et al., 2013), although different receptors seem to be involved. In neuronal and probably in different erythroid cell types, Epo may act through the classical homodimeric (EpoR/EpoR) and the heterodimeric (EpoR/βcommonR) receptors, very likely depending on the relative density of each subunit. In contrast, cEpo may only bind to the heterodimer which comprises an EpoR subunit and a βcommon subunit (Chamorro et al., 2013), the latter being also shared with the GM-CSF, IL-3 and IL-5 receptors.

The finding of a differential action between Epo and cEpo on cell proliferation signaling in erythroid cells was highlighted in our previous work (Chamorro et al., 2013). Therefore, we decided to investigate this point further using the UT-7 cell line, which is fully dependent on Epo to survive, as an erythroid model. In cEpo-stimulated cultures, we found a relationship between the dephosphorylation rate of different factors in signaling pathways related to cell proliferation and PTP1B activation.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. Dulbecco's modified Eagle medium (D-MEM), Ham F12 medium, and Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum (FBS, Bioser) and penicillin–streptomycin were obtained from GibcoBRL. Aprotinin, leupeptin, pepstatin A, sodium *o*-vanadate, phenylmethylsulfonyl fluoride (PMSF), L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and luminol were obtained from Sigma-Aldrich; Agarose protein A, Cytofix/Cytoperm and Perm Wash were from BD Transduction Laboratories; Alexa Fluor 488 and Alexa Fluor 594 labeled secondary antibodies were from Invitrogen Life Technologies; anti β-actin (sc-47778), anti EpoR (M-20 sc-697), anti βcommon (IL-3/IL-5/GM-CSFRβ sc-21765), anti Jak2 (sc-294), anti PTP1B (sc-14021), anti ERK (sc-292838), anti phosphorylated ERK (sc-136521) and CinnGel 2ME were from Santa Cruz Biotechnology. Anti phosphorylated FOXO3a, anti FOXO3a, anti phosphorylated Akt, anti Akt and anti phosphorylated Jak2 antibodies were from Cell Signaling Technology. Nitrocellulose (NC) membranes (Hybond) were from Amersham Bioscience; acrylamide/bis-acrylamide solution, sodium *p*-nitrophenylphosphate and potassium cyanate were from Merck. Recombinant human erythropoietin (rhEpo) was kindly supplied by Zelltek (Argentina).

2.2. Preparation of cEpo

Carbamylated erythropoietin (cEpo) was prepared as described by Leist et al. (2004) with modifications (Chamorro et al., 2013). Briefly, one volume of Epo (0.5 mg/mL) was mixed with one volume of 1 M sodium borate (pH 8.8) and one volume of 3 M potassium cyanate (KCNO). The mixture was incubated at 37 °C for 48 h. Samples were dialyzed against Milli-Q water during 72 h (4 °C) with frequent changes of liquid, and then concentrated by Centricon (Millipore, 10 kDa cut-off). The decrease in the number of free amino groups measured by its reaction with 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1966) was used to determine the efficiency of carbamylation.

Since carbamylation of Epo leads to the transformation of lysine residues into homocitrulline, the homogeneity of the preparation was controlled by gel electrophoresis under alkaline non-denaturing conditions and immunoblotting, and it displayed an increased electrophoretic mobility of cEpo with respect to Epo. This is explained by the increase in net electric charge of the molecule. Similarly, in capillary electrophoresis cEpo gave a single peak with a higher retention time than Epo.

The same batch of cEpo was used throughout the work after demonstration of its lack of proliferative action on UT-7 cells as well as its protective effect on SH-SY5Y cells induced to apoptosis by staurosporine.

2.3. Cell lines and cultures

(a) The human UT-7 cell line, kindly provided by Dr. Patrick Mayeux (Cochin Hospital, Paris, France), shows growth dependence on Epo. These cells were maintained in IMDM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 U/mL Epo (Vittori et al., 2005).
(b) Human SH-SY5Y neuroblastoma cells (CRL-2266, ATCC) were grown in 5 mL of 1:1 D-MEM:Ham F12 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% (v/v) heat-inactivated FBS (Pregi et al., 2006; Wenker et al., 2010; Chamorro et al., 2013).

Cell cultures were developed at 37 °C in an atmosphere containing 5% CO_2 and 100% humidity. The medium was routinely replaced every 2–3 days. Cell viability and proliferation were evaluated by Trypan blue exclusion test and the MTT assay.

2.4. MTT assay

Cells were cultured in 35 mm-Petri dishes at a density of 2×10^5 cells/mL. After the cells had been subjected to appropriate treatments and the medium removed, they were incubated at 37 °C with MTT at a final concentration of 0.5 mg/mL. The supernatant was removed and the pellet washed with phosphate buffer saline (PBS). Finally, 100 μL of 0.04 M HCl in isopropanol were added to dissolve the blue formazan product (reduced MTT), which was quantified by measuring absorbance at 570 nm test wavelength and 655 nm reference wavelength in a microplate reader (BioRad).

2.5. Electrophoresis and Western blotting

Cells were washed with ice-cold PBS and lysed with a hypotonic buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM sodium *o*-vanadate) containing protease inhibitors (1 mM PMSF, 4 μM leupeptin, 1 μg/mL aprotinin, 2 μM pepstatin), in a ratio of 200 μL/10^7 cells. After 30 min of incubation on ice, insoluble material was removed by centrifugation (15,000 × *g*, 15 min). Cell extracts were boiled for 3 min in the Laemmli sample buffer (Laemmli, 1970) and resolved by SDS–polyacrylamide gel electrophoresis (T 10%). Electrophoresis under non-denaturing conditions was performed in a Miniprotean III electrophoretic system (BioRad) using 10% polyacrylamide gels. After electrophoresis, protein samples were electroblotted onto a nitrocellulose membrane for 1.5 h (transfer buffer: pH 8.3, 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% (v/v) methanol). Membranes were blocked by 1 h-incubation in Tris Buffer Saline (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 specific antibodies. After washing with TBS-0.1% Tween 20, the immunoblots were probed with adequate peroxidase-conjugated secondary antibodies (1:1000) for 1 h at 20 °C and washed. Antigen-antibody complex signals were

detected by enhanced chemiluminiscence, using luminol and a Fujifilm Intelligent Dark Box II equipment (Fuji) coupled to a LAS-1000 digital camera. Anti-β-actin polyclonal antibody was used to assess sample loading variations. Densitometry with GelPro software was performed to quantify the bands.

2.6. Jak2 phosphorylation and PTP1B expression by flow cytometry

After experimental treatments, cells were collected by centrifugation and washed with 2% FBS in PBS. Cells were then incubated with Cytofix–Cytoperm for 20 min, and following centrifugation (350 × *g*, 10 min) they were incubated with anti phosphorylated Jak2 or anti PTP1B antibodies (30 min, 4 °C) and washed with Perm Wash. After that, samples were incubated with an Alexa Fluor 488-bound secondary antibody (30 min, 4 °C) and finally analyzed in a flow cytometer equipped with a 488 nm argon laser (FACSort, Becton-Dickinson). WinMDI 2.9 software was used to analyze data.

2.7. Enzyme activity assay

2.7.1. Protein immunoprecipitation

Anti PTP1B antibody was added at 2 μg/mL final concentration to cell extracts obtained as explained in Section 2.5, which were then incubated at 4 °C for 1 h with gentle agitation. Protein A-agarose was added and, after 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.

2.7.2. PTP1B activity

Aliquots of cell lysate equivalent to 1 mg total protein were immunoprecipitated with polyclonal anti PTP1B and immunoprecipitates washed with PTP1B assay buffer (18.5 mM HEPES, pH 7.2, 7.5 mM NaCl, 1.85 mM EDTA, 5 mM DTT). PTP1B activity was measured by incubation at 37 °C for 30 min in PTP1B assay buffer containing 2.4 mM pNPP, in a final volume of 200 μL. Optical density of the product 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 the enzyme (Callero et al., 2011).

2.8. Confocal microscopy

Cells (5×10^5) were washed twice with 2% FBS in PBS, fixed and permeabilized for 20 min at 4 °C with Cytofix/Cytoperm. They were then washed with Perm Wash. The pellet was incubated with anti PTP1B, anti βRc or anti EpoR for 30 min at 4 °C. After washing with Perm Wash, they were incubated with Alexa Fluor 488 and Alexa Fluor 594 secondary antibodies for 30 min at 4 °C. Following further washing, the preparations were mounted on polylysine slides and covered to be observed through a confocal microscope with Argon 488 and HeNe Green 543 lasers. The images were analyzed with the acquisition software Olympus Fluorview 5.0.

2.9. Statistics

Results are expressed as mean ± standard error of the mean (mean ± SEM). 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.

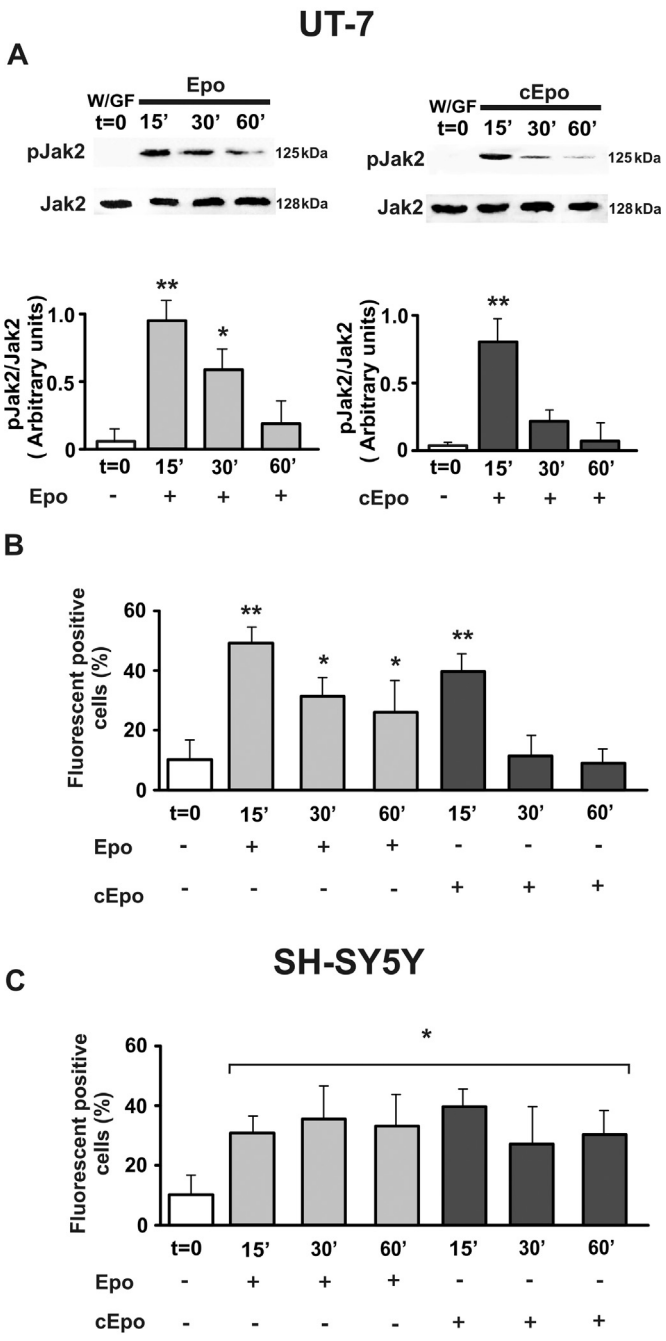

Fig. 1. Time-dependent Jak2 activation induced by Epo and cEpo. After 18 h-starvation ($t = 0$), UT-7 cells were incubated during 15, 30, or 60 min with 20 ng/mL Epo or 20 ng/mL cEpo. At the end of each period, Jak2 phosphorylation was determined by Western blotting (A) and flow cytometry (B). (A) Cell extracts were resolved by SDS–PAGE ($T = 10\%$), electroblotted onto a NC membrane and then immunodetected by chemiluminescence using specific antibodies. Detection of non phosphorylated Jak2 was used as protein loading control. Western blots are representative of 3 independent assays and bars indicate the ratio of band density between phosphorylated and non phosphorylated proteins. Significant differences: Epo15′ or cEpo15′ vs. $t = 0$, ** $P < 0.01$; Epo30′ vs. $t = 0$, * $P < 0.05$; cEpo30′ vs. $t = 0$, NS ($n = 3$). (B) Anti pJak2 as primary antibody and an Alexa Fluor 488-bound secondary antibody were used for flow cytometry analysis. Each bar represents mean ± SEM of fluorescent pJak2-positive cells. Significant differences: Epo15′ or cEpo15′ vs. $t = 0$, ** $P < 0.01$; Epo30′ or Epo 60′ vs. $t = 0$, * $P < 0.05$; cEpo30′ or cEpo 60′ vs. $t = 0$, NS ($n = 3$). (C) SH-SY5Y cells were cultured for 48 h to reach confluence. At this time ($t = 0$), they were incubated with 250 ng/mL of either Epo or cEpo and Jak2 phosphorylation was analyzed by flow cytometry at each time points indicated in the figure. Significant differences: * $P < 0.05$ vs. $t = 0$ ($n = 3$). cEpo treatments vs. Epo treatments, NS.

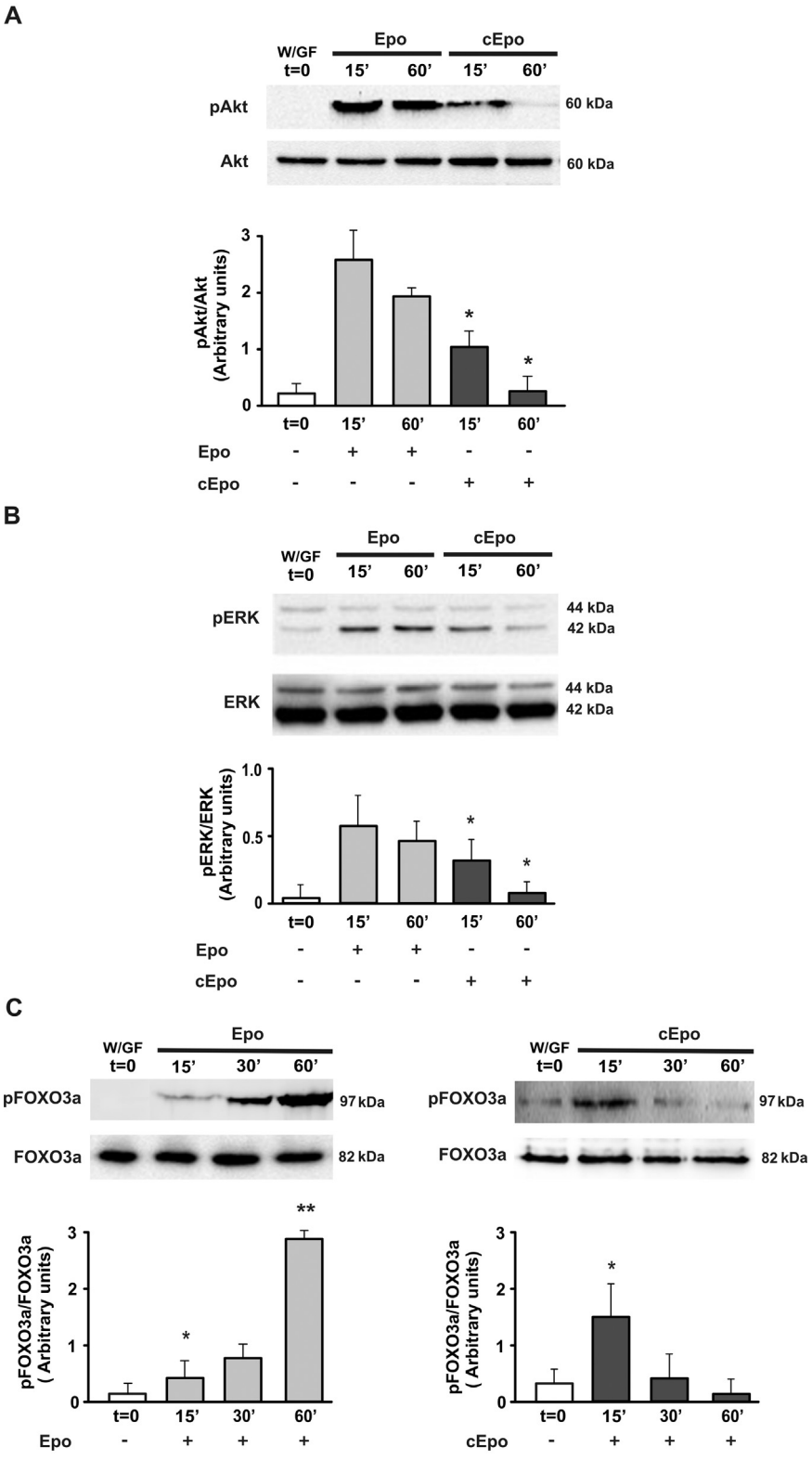

Fig. 2. Time-dependent dephosphorylation in cell proliferation signaling induced by Epo and cEpo. UT-7 cells were cultured without growth factors for 18 h (W/GF) and then incubated with 20 ng/mL of either Epo or cEpo for different periods. Cell lysates were analyzed by SDS–PAGE and immunoblotting with specific primary antibodies: anti pAkt and anti Akt (A), anti pERK1/2 and anti ERK1/2 (B) or anti pFOXO3a and anti FOXO3a (C). Reactions were followed by incubation with the appropriate horseradish peroxidase-bound secondary antibody and chemiluminiscence detection. The photographs shown are representative of 3 independent assays. Bars indicate the ratio of band density between phosphorylated and non phosphorylated proteins (In B, p42 kDa vs. 42 kDa ERK protein). Significant differences: (A and B) cEpo 60′ vs. Epo 60′ and cEpo 15′ vs. Epo 15′, * $P < 0.05$. (C) Epo 15′ or cEpo 15′ vs. $t = 0$, * $P < 0.05$; Epo 60′ vs. $t = 0$, ** $P < 0.01$; cEpo60′ vs. $t = 0$, NS.

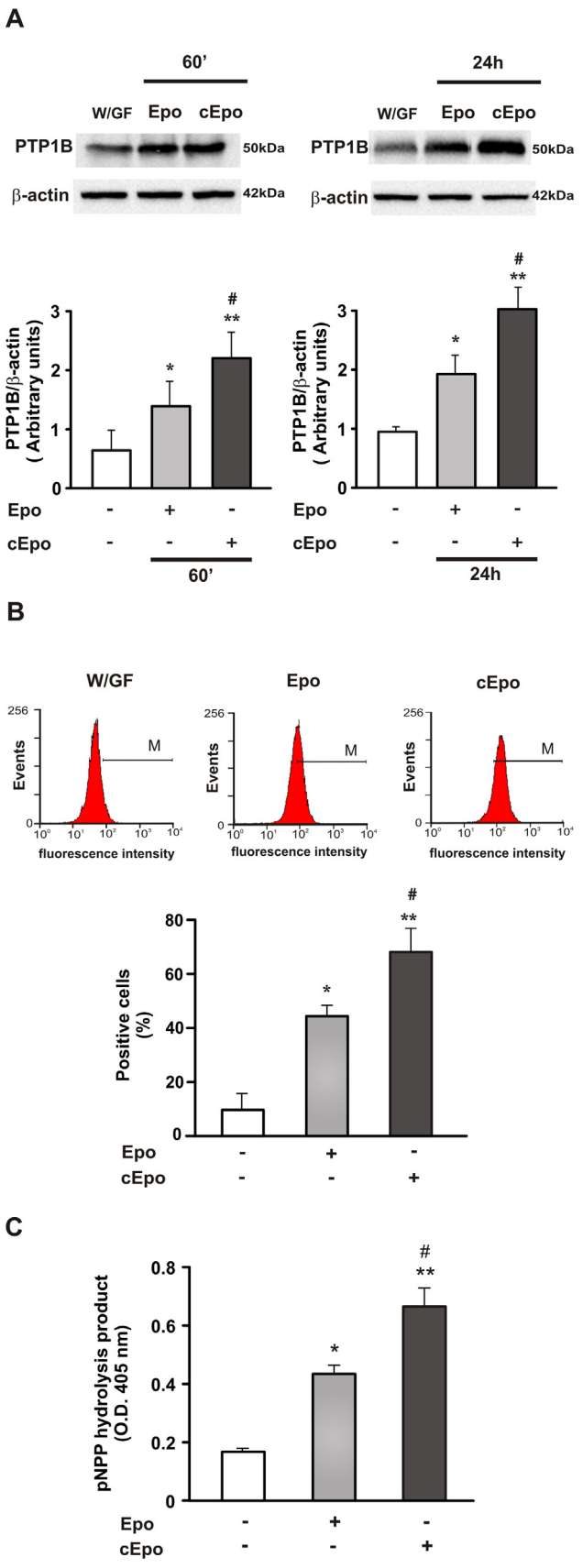

Fig. 3. PTP1B expression and activity induced by Epo and cEpo. (A) UT-7 cells were cultured without growth factors for 18 h (W/GF) and then incubated in the presence of 20 ng/mL of either Epo or cEpo for 60 min or 24 h. Cell lysates were analyzed by SDS–PAGE and immunoblotting revealed by chemiluminiscence. Blots are representative of 4 assays with similar results. Bars indicate band density of PTP1B with respect to β-actin (protein loading control). (B) UT-7 cells treated with Epo or cEpo as indicated above were sequentially exposed to anti PTP1B and to a secondary antibody bound to Alexa Fluor 488, to analyze PTP1B expression by flow cytometry. Histograms are representative of 3 independent assays and bars indicate percentage of fluorescent PTP1B-positive cells. (C) After 18 h W/GF, UT-7 cells were treated with Epo (20 ng/mL) or cEpo (20 ng/mL) for 2 h. Cell lysates were immunoprecipitated with anti PTP1B antibody and phosphatase activity was determined in the pellet by measuring absorbance of the pNPP hydrolysis product at 405 nm ($n = 4$). Significant differences: cEpo vs. W/GF, ** $P < 0.01$; Epo vs. W/GF, * $P < 0.05$, cEpo vs. Epo, # $P < 0.05$.

3. Results

3.1. Time dependence of Jak2 phosphorylation by Epo and cEpo

As we have previously reported, cEpo was unable to trigger erythroid cell proliferation, yet it induced Jak2 phosphorylation in the UT-7 and TF-1 cell lines, which are known to be activated by Epo (Chamorro et al., 2013). Since this effect was detected after a 10 min-treatment of cell cultures with Epo, we decided to analyze a possible time-dependent induction of Jak2 phosphorylation by cEpo and compare it with that induced by Epo. We found a prolonged activation of Jak2 in cell cultures stimulated by Epo in comparison with those performed in the presence of its derivative (Fig. 1). Even though Jak2 phosphorylation was significantly induced by both erythropoietins at 15 min, activation by cEpo rapidly disappeared being barely detectable at 30 min (Fig. 1A and B). It is worth mentioning that both Epo and cEpo maintained Jak2 phosphorylation at similar levels at least up to 60 min in SH-SY5Y cell cultures (Fig. 1C). This was an expected result, since both erythropoietins are known to have similar neuroprotective behavior (Chamorro et al., 2013).

3.2. Time-dependent dephosphorylation of cell proliferation signaling induced by cEpo

Taking into account that the kinetics of Jak2 phosphorylation differed when induced by Epo or cEpo, we decided to investigate whether this could lead to a time-dependent dephosphorylation of downstream targets related to cell proliferation, such as Akt and Erk1/2. Therefore, we analyzed the phosphorylation of these factors at different times of cell activation.

In UT-7 cells, Akt phosphorylation was strongly induced after 15 min of Epo treatment, and remained high after 60 min. On the contrary, it was significantly lower at 15 min and barely detectable after 60 min of exposure to cEpo. (Fig. 2A). Similar results were observed for the time course of ERK1/2 phosphorylation (Fig. 2B). This way, the active Epo signaling pathway went on leading to FOXO3a phosphorylation, thus preventing the expression of the cell cycle inhibitor $p27^{kip1}$, which was previously reported (Chamorro et al., 2013), while pFOXO3a rapidly decreased in the presence of cEpo (Fig. 2C).

3.3. PTP1B expression and activity induced by Epo and cEpo

Phosphatases play a key role in the "turning off" of signals activated by protein kinases. Here, we demonstrate that although cEpo is able to induce phosphorylation of factors known to be activated by Epo, they are rapidly dephosphorylated. These results suggest that an increased phosphatase activity may be involved in cEpo stimulation. Apart from modulating Epo/EpoR signaling, PTP1B undergoes feedback regulation by Epo (Callero et al., 2007), which prompted us to investigate the induction and/or interaction of PTP1B with cEpo. We analyzed PTP1B protein expression in UT-7 cells cultured in the presence of Epo or cEpo.

PTP1B expression was immunodetected by Western blotting (Fig. 3A) and by flow cytometry (Fig. 3B) after cells were exposed

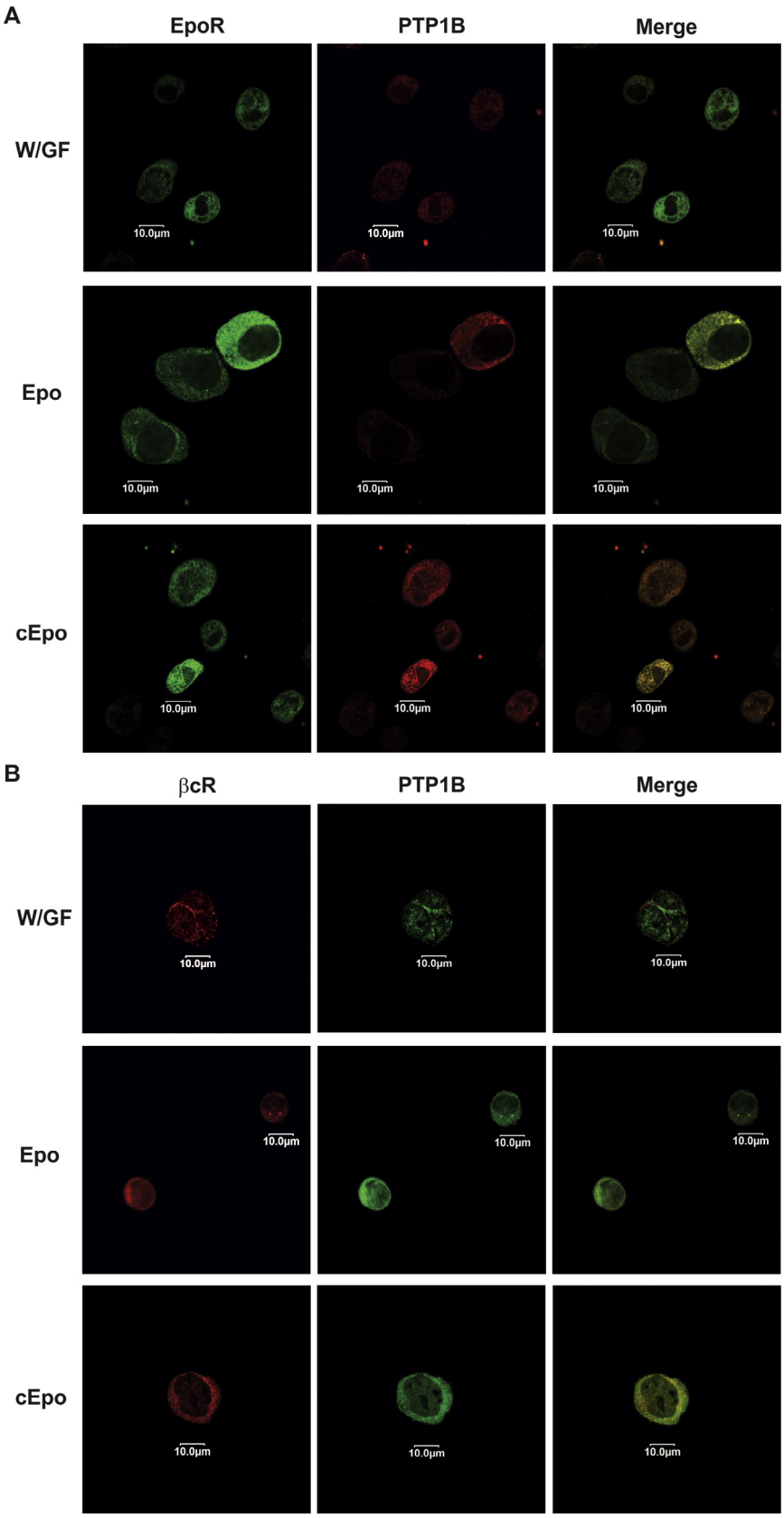

Fig. 4. Colocalization of PTP1B with EpoR and βcommon subunits. UT-7 cells were grown without stimulating factors for 18 h and then incubated for 10 min without growth factors or with either Epo (20 ng/mL) or cEpo (20 ng/mL). Specific primary antibodies (anti PTP1B, anti EpoR and anti βcR) and secondary antibodies bound to Alexa Fluor 488 or Alexa Fluor 594 were used. Confocal microscopy images were taken with 1500x magnification. Yellow staining represents colocalization between PTP1B (red) and EpoR (green) or between PTP1B (green) and βcR (red). The scale bar is indicated in the figure.

to each erythropoietin. Interestingly, the results showed not only the expected modulation of PTP1B by Epo, but also a significantly higher level of PTP1B induced by cEpo.

In order to elucidate if cEpo can stimulate the activity of this phosphatase as well as its expression, we further analyzed PTP1B enzymatic activity in UT-7 cell immunoprecipitates. As expected, PTP1B activity – measured as pNPP hydrolysis – was enhanced in cultures with Epo, yet it was significantly higher in the presence of cEpo (Fig. 3C). These results parallel those related to PTP1B protein expression.

3.4. Colocalization of PTP1B and the βcommon receptor

The results up to here show the action of cEpo as an inducer of PTP1B expression and enzymatic activity. In a previous work, we concluded that Epo is able to interact with cells showing neuronal characteristics, and probably with erythroid cells as well, through both receptors, the homodimer (EpoR/EpoR) and the heterodimer (EpoR/βcR), whereas cEpo can only bind to the heterodimer. After demonstrating the expression of the heterodimeric receptor in UT-7 cells, we continued to investigate a possible relationship between PTP1B and the βcommon subunit by confocal microscopy.

Fig. 4 shows colocalization of PTP1B with βcR and EpoR in UT-7 cells induced by cEpo. High intensity of colocalization was observed between PTP1B and EpoR in Epo-treated cells whereas no colocalization was detected in the absence of growth factors.

3.5. Cell proliferation in the presence of phosphatase inhibitors

The novel result in this work showing significant increase of PTP1B expression and activity induced by cEpo in UT-7 cells may contribute to explain the early dephosphorylation of factors involved in cell proliferation signaling pathways and therefore, the inability of the carbamylated protein to act as growth factor for cells capable of erythroid differentiation. The following step was then to evaluate cell response to cEpo activation in the presence of phosphatase inhibitors.

Assays were performed in the presence of the non-specific phosphatase inhibitor sodium *o*-vanadate, or CinnGel 2ME. The latter, a selective inhibitor of PTP1B, is an ester which can be hydrolyzed by intracellular esterases, yielding cinnamic acid (Fig. 5). As expected, proliferation of cEpo-treated cultures was similar to those carried out without growth factors. The figure shows that Epo-induced cell proliferation significantly increased in the presence of CinnGel 2ME with respect to cultures in the absence of inhibitor. Preincubation with CinnGel 2ME allowed a significant increase in cell proliferation after cEpo treatment. Although it failed to match the increment induced by Epo, it still exceeded the values detected with cEpo alone (Fig. 5A). The effects of the non-specific phosphatase inhibitor *o*-vanadate on cultures are shown in Fig. 5B. It appears that the non-specific inhibition of phosphatase activity makes cEpo more capable of inducing proliferation of the UT-7 cell line.

The results suggest that PTP1B might – at least in part – be responsible for the inability of cEpo to act as a growth factor, even though the overexpression of other phosphatases should not be disregarded.

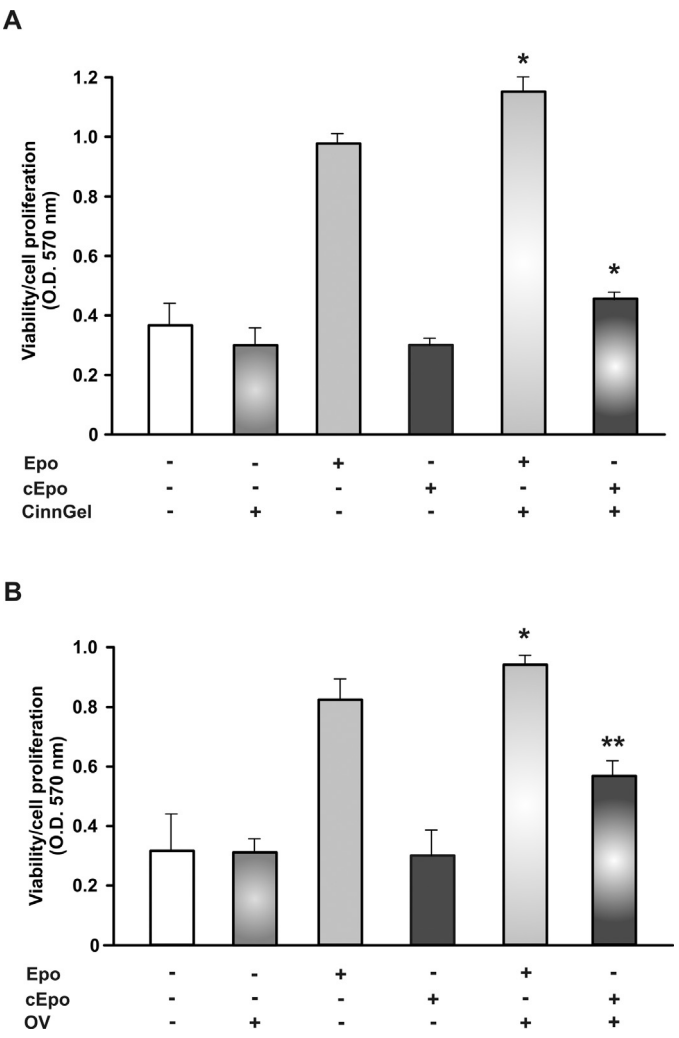

Fig. 5. Cell proliferation in the presence of phosphatase inhibitors. Following 18 h-deprivation of growth factors, UT-7 cells were grown in the presence of 10 μM CinnGel 2ME (A) or 0.5 mM sodium *o*-vanadate (B) for 2 h. After addition of Epo (20 ng/mL) or cEpo (20 ng/mL) cultures were carried out for additional 48 h. Cell viability/proliferation was evaluated by the MTT assay and absorbance was measured at 570 nm (reference λ: 655 nm). Significant differences: Epo + CinnGel vs. Epo or cEpo + CinnGel vs. cEpo, * $P < 0.05$; Epo + OV vs. Epo, * $P < 0.05$; cEpo + OV vs. cEpo, ** $P < 0.01$ ($n = 3$); CinnGel or OV vs. Control, NS.

4. Discussion

Much research agrees with the concept that carbamylation of Epo lysine residues leads to a derivative that retains the ability of the native protein to protect neuronal cells against apoptosis while it is unable to support proliferation of erythroid cells. Regarding SH-SY5Y neuroblastoma cells, we reported similar Jak2 and PI3K-mediated mechanisms for the anti apoptotic actions of Epo and cEpo (Chamorro et al., 2013) in accordance with Xu et al. (2009) who found that in a rat model, the PI3K/Jak2 signaling pathway was involved in cardioprotection by carbamylated erythropoietin *in vivo*. Interestingly, in that previous work we observed that cEpo also induced Jak2 phosphorylation in UT-7 and TF-1 cells with ability for erythroid differentiation despite the protein inability to act as a growth factor for these cell lines. Furthermore, in cell cultures with cEpo cell cycle progression was restrained at the G1/S transition in line with the findings of non phosphorylated forkhead factor FOXO3a and upregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$. These results let us then suggest that the main difference between Epo and cEpo in cell cultures capable of erythroid differentiation was related to cell proliferation and we decided to go further into this topic by investigating possible differences in the phosphorylation time course of each factor involved in cell proliferation pathways.

The differential effects of Epo and cEpo have been ascribed to their binding to different receptors, the homodimer $(EpoR)_2$ and the heterodimer EpoR/βcommonR, respectively (Brines et al., 2004; Xu et al., 2009). Nevertheless, this point seems not to be relevant to explain the different action between Epo and its carbamylated

derivative since Jak2 phosphorylation induced by cEpo suggests that the interaction between cEpo and erythroid cells involves a receptor belonging to the type I cytokine superfamily. Regardless of which receptor – $(EpoR)_2$ or EpoR/βcR – is engaged in the ligand/receptor interaction, the first sign is the increased tyrosine phosphorylation and activation of the receptor-associated protein Jak2. This kinase mediates the subsequent phosphorylation of tyrosine residues not only within the EpoR but also within the β-chain of the heteroreceptor which is a subunit also shared with the GM-CSF, IL-3 and IL-5 receptors. In the present work, we found that even though Jak2 phosphorylation was induced by both erythropoietins at 15 min, activation by cEpo rapidly disappeared, being undetectable at 30 min, while Epo prolonged Jak2 activation at least up to 60 min (Fig. 1).

Similar results were observed for the phosphorylation time course of other factors involved in signaling pathways related to cell proliferation. Akt and ERK1/2 phosphorylation was strongly induced after 15 min of Epo treatment, and remained high after 60 min. On the contrary, Akt and ERK phosphorylation induced by cEpo was significantly lower at 15 min and barely detectable at 60 min. FOXO3a phosphorylation increased in a time-dependent manner due to Epo activation, while rapidly decreased in the presence of cEpo (Fig. 2). Thus, dephosphorylated FOXO3a translocates to the nucleus, and consequently stimulates the expression of $p27^{kip1}$, known as a key factor responsible for the negative modulation of cell cycle and cell proliferation (Nakao et al., 2008; Chamorro et al., 2013).

Our results agree with those of Ramirez et al. (2009) who detected Jak2/Akt phosphorylation in UT-7 cell cultures but a very weak effect of carbamylated darbepoietin on proliferative signals (ERK1/2, NF-kB and STAT-5) of endothelial progenitors.

It is known that phosphorylation of proteins at tyrosine residues serves as a regulatory switch in different biological processes and cellular events. Tyrosine phosphorylation is controlled by the coordinated actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). These two families of enzymes maintain a dynamic balance of phosphotyrosine residues that are crucial for cellular homeostasis. To date, the family of PTPs which includes over 100 members (Alonso et al., 2004; Andersen et al., 2004), has been shown to regulate many cellular events such as differentiation, cell growth, motility, and proliferation (Tonks and Neel, 2001). The widely expressed PTP1B is the prototype for the superfamily of PTPs, and localizes to the endoplasmic reticulum (ER). Several studies have implicated PTP1B in the attenuation of various PTKs signaling pathways, namely those of the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR), the insulin receptor (IR) and the erythropoietin receptor (EpoR) (Kenner et al., 1996; Myers et al., 2001; Buckley et al., 2002; Gu et al., 2003; Haj et al., 2003; Callero et al., 2007), thus involving PTP1B in the regulation of a variety of cellular events (Dube and Tremblay, 2004). It can be deduced from these reports that PTP1B overexpression can diminish signaling from these kinases. In this context, we hypothesized that overexpression of PTP1B could be involved in the rapid dephosphorylation of proliferative signals observed in UT-7 cell cultures with cEpo. Indeed, we found not only significantly higher levels but also higher activity of this phosphatase in cultures exposed to cEpo with respect to those activated by Epo (Fig. 3). Besides, the interaction between PTP1B and the subunits EpoR and βcommonR, components of the heteroreceptor used by cEpo (Fig. 4), provides further evidence of the role of this phosphatase in the decreased erythropoietic activity of cEpo.

Taking these results into consideration, we assumed that overcoming phosphatase activity may let cEpo induce cell proliferation. To study this point, we used two phosphatase inhibitors, the unspecific sodium *o*-vanadate and the specific PTP1B inhibitor CinnGel 2ME. Although treatment with cEpo in the presence of CinnGel 2ME was not sufficient to reach the cell proliferation levels observed in Epo-induced cultures, it still exceeded the values detected with cEpo alone. However, it appears that the non-specific inhibition of phosphatase activity makes cEpo more capable of inducing proliferation of the UT-7 cell line (Fig. 5). Here we report that PTP1B as well as other phosphatases may play a role in the inability of cEpo to induce erythroid cell proliferation. Nonetheless, further investigation is needed to clarify why cEpo is inactive, given that the upregulation of PTPs, which is induced by this modified protein, fails to provide a complete explanation. In this context, it is known that activation of EpoR after Epo binding is transient as EpoR appears to be quickly degraded after ubiquitination by two proteolytic systems that act sequentially: the proteasomes removing part of the intracellular domain at the cell surface, and the lysosomes degrading the remaining part of the receptor–hormone complex (Walrafen et al., 2005). There is a possibility that these systems could also be activated by cEpo after binding to the EpoR/βcR.

5. Conclusions

In this study we have demonstrated for the first time that the catalytic activity of PTP1B and probably other phosphatases appears necessary to deprive cEpo of erythropoietic function. We believe that the present results open interesting research areas concerning the potential role of tyrosine-phosphatases in controlling growth factor functions.

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