Protein tyrosine phosphatase 1B participates in the down-regulation of erythropoietin receptor signalling

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Erythropoietin (EPO) is the principal hormone regulating the proliferation of erythroid precursors and their differentiation into erythrocytes. Binding of ligand to the cell-surface EPO-R (EPO receptor) induces dimerization and JAK2 (Janus kinase 2)-mediated tyrosine phosphorylation of the receptor. Less than 1 % of the EPO-Rs are displayed on the cell surface; most of the receptor molecules are retained in intracellular compartments, including the ER (endoplasmic reticulum). Using pervanadate (PV) as a potent tool to inhibit cellular PTPs (protein tyrosine phosphatases), we demonstrated previously the accumulation of mature (endoglycosidase H-resistant) tyrosine-phosphorylated EPO-R [Cohen, Altaratz, Zick, Klingmuller and Neumann (1997) *Biochem. J.* **327**, 391–397]. In the present study, we investigated the participation of the ER-associated PTP1B in the dephosphorylation of intracellular EPO-R. We demonstrate tyrosine phosphorylation of EPO-R in BOSC-23T cells co-expressing EPO-R

and the 'substrate-trapping' mutant form of PTP1B, PTP1B D181A (referred to as PTP1BD). *In vivo* interaction between EPO-R and PTP1B suggested that PTP1B dephosphorylates the EPO-R intracellularly. Endoglycosidase H resistance of tyrosine-phosphorylated EPO-R in cells expressing PTP1BD suggested that mature EPO-R is dephosphorylated by PTP1B. Stimulation with EPO of cells co-expressing EPO-R and either PTP1BD or PTP1B resulted in an increase or decrease respectively in phosphotyrosine EPO-R. We thus suggest that PTP1B dephosphorylates EPO-stimulated EPO-R and participates in the down-regulation cascade of EPO-mediated signal transduction.

Key words: erythropoietin receptor, Janus kinase 2, PTP1B (protein tyrosine phosphatase 1B) 'substrate-trapping' mutant, tyrosine phosphorylation.

INTRODUCTION

Proliferation of erythroid progenitor precursor cells and their differentiation into mature erythrocytes are mediated by the hormone erythropoietin (EPO) via its cell-surface receptor. EPO-R (EPO receptor) belongs to the cytokine receptor superfamily, which shares common structural motifs: one transmembrane domain, four cysteine residues similarly spaced and a common sequence motif WSXWS in the extracellular domain [1]. Unlike receptor tyrosine kinases, the cytosolic domain of cytokine receptors is devoid of kinase activity. Binding of ligand to cell-surface EPO-R results in the activation of JAK2 (Janus kinase 2), which phosphorylates the receptor [2,3]. Tyrosine-phosphorylated EPO-R then serves as a docking site for other signalling proteins, which, in synchrony with JAK2, mediate the cellular response to EPO. Structural analysis led to the proposal that unliganded EPO-R exists as a preformed homodimer in an open scissor-like conformation [4] and that dimerization induced by the transmembrane domain maintains unliganded EPO-R in an inactive state. EPO in physiological amounts can then switch the receptor to an activated state [5,6]. Termination of EPO-R signalling is mediated by the interaction of selected proteins with the EPO-R or with signalling molecules. A major player in such interaction is the haematopoietic PTP (protein tyrosine phosphatase) SH-PTP1 (also referred to as HCP or PTP1C), which is recruited via its SH2 (Src homology 2) domains to pTyr (phosphotyrosine)-429

of the EPO-R, leading to dephosphorylation of JAK2 and down-modulation of positive signals [3].

Treatment of cells with pervanadate (PV) inhibits PTPs, thereby perturbing the basal equilibrium of tyrosine phosphorylation/dephosphorylation and culminating in increased protein tyrosine phosphorylation [7,8]. Since PV can easily penetrate the cell membrane, it serves as a unique tool to activate both surface and intracellular forms of the EPO-R, as opposed to stimulation with EPO, which allows the analysis of only cell-surface-derived EPO-R. Using this approach, we demonstrated previously that tyrosine phosphorylation of the EPO-R might also occur in the ER (endoplasmic reticulum) [9] and that the EPO-R might assemble with JAK2 while in the ER. Recent evidence points to an interaction of JAK2 with newly synthesized EPO-R in the ER, apparently necessary for the efficient expression of the receptor on the cell surface [10]. Recently, JAK2 was implicated in the modulation of the degradation of intracellular pools of growth hormone receptor [11], which (similar to the EPO-R) is a member of the cytokine receptor superfamily [12].

These findings raise fundamental questions concerning possible interactions between EPO-R and protein tyrosine kinases and PTPs that are associated with the ER. Accumulating evidence indicates that intracellular membranes harbour signalling molecules. For example, Src-homology collagen (Sch) proteins were localized in the ER and were found, after tyrosine kinase receptor activation, to redistribute to the cytosolic surface of the plasma

Abbreviations used: EGF-R, epidermal growth factor receptor; endo H, endoglycosidase H; EPO, erythropoietin; EPO-R, EPO receptor; ER, endoplasmic reticulum; GFP, green fluorescent protein; GST, glutathione S-transferase; JAK, Janus kinase; PTP, protein tyrosine phosphatase; pTyr, phosphotyrosine; PV, pervanadate; STAT5a, signal transducer and activator of transcription 5a; TCPTP, T-cell PTP; wt, wild-type.

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membrane and endocytic structures [13]. The ER tyrosine kinase Ltk was identified and its activity was found to be regulated by the redox potential within the ER [14]. Recent results point to the activation of Ras in association with ER membranes [15]. PTP1B is a widely expressed non-receptor PTP, located on intracellular membranes via its hydrophobic C-terminal targeting sequence [16]. It was suggested that PTP1B plays a central role in the regulation of many cellular functions including cell adhesion (see e.g. [17,18]), response to cellular stress and cytokine receptor signalling [19–21].

To identify potential physiological substrates of PTP1B, 'substrate-trapping' mutants were developed [22]. This approach exploits the ability of certain PTP mutants to bind to their substrates, but not to dephosphorylate them. Such PTP1B mutants with decreased catalytic activity were shown to form complexes with their intracellular substrates and to lead to the accumulation of their respective pTyr forms by protecting these proteins from dephosphorylation by endogenous PTP1B. Substrates of PTP1B include EGF-R (epidermal growth factor receptor) [22], platelet-derived growth factor receptor [23], insulin receptor [24], JAK2 [19] and STAT5a (signal transducer and activator of transcription 5a) and STAT5b [21]. Until recently, PTP1B was believed to associate with and dephosphorylate ER-associated substrates. Recent reports implicate PTP1B in the regulation of ligand-mediated phosphorylation of receptor tyrosine kinases [25,26]. In this context, it is worth noting that in the presence of the substrate-trapping form of PTP1B, tyrosine phosphorylation of JAK2 was increased after ligand stimulation [19], suggesting that PTP1B participates in the down-regulation of tyrosine kinases associated with cytokine receptor signalling. Thus, the central role of PTP1B is inferred by its effects on both ligand-independent and -induced events as well as on the assembly of ER *in vitro* [27].

The physiological response to IL-3 (interleukin-3) is attenuated when IL-3-dependent 32D cells are transfected with the cDNA of PTP1B [28]. The above observations, together with the likelihood that the EPO-R and the IL-3 receptor utilize similar intracellular signalling cascades [29] and the ability of the EPO-R to undergo tyrosine phosphorylation [9] while residing in the ER, prompted us to examine whether the EPO-R is a substrate of PTP1B.

We show here that EPO-R interacts with PTP1B and the substrate-trapping mutant form of PTP1B, PTP1B D181A (referred to as PTP1BD). We demonstrate that whereas unprocessed EPO-R interacts with both PTP1B and PTP1BD, only endo H (endoglycosidase H)-resistant EPO-R (which comprises a minute fraction of the receptor molecules) is tyrosine-phosphorylated in cells co-expressing the EPO-R and PTP1BD, suggesting that PTP1B dephosphorylates mature EPO-R. The increased tyrosine phosphorylation of the EPO-R in cells co-expressing the EPO-R and PTP1BD after stimulation with EPO and the decreased tyrosine phosphorylation of the EPO-R in EPO-responsive cells co-expressing the EPO-R and PTP1B suggest that PTP1B plays a role in the down-regulation of ligand-activated EPO-R.

MATERIALS AND METHODS

Materials

All materials were obtained from sources listed previously [9,30]. PV was generated as described previously [9].

Antibodies

Rabbit antibodies directed against the N-terminus of EPO-R [30] or against a GST (glutathione S-transferase)-fusion protein containing either the cytosolic [31] or the extracellular domain of mouse EPO-R [3] were used at dilutions of 1:250 for immuno-

precipitation and 1:1000 for immunoblotting. Rabbit antibodies directed against the C-terminal peptide of the EPO-R [32] were used at a dilution of 1:1000 for immunoblotting of GST-phosphatase-precipitated EPO-R. A monoclonal antibody against pTyr (PY), 4G10 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), was employed. Mouse monoclonal antibodies against PTP1B (FG6 [22]) and TCPTP (T-cell PTP) [33] were a gift from Professor N. Tonks (Cold Spring Harbor Laboratory, Plainview, NY, U.S.A.).

Cells and plasmids

BOSC-23T cells [34] were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum. Cells (60 % confluent) were transiently transfected using the calcium phosphate method [35] and were harvested 48 h after transfection. Transfection was performed (using 10 µg of each plasmid) with pXM EPO-R cDNA and pMT2 PTP1B or (GST)-PTP1B-fusion protein in pMT2 (GST tag, N-terminus) or PTP1BD in these pMT2 vectors [22]. PTP cDNA constructs were generously provided by Professor N. Tonks. Plasmid pNeoSRaII, carrying full-length human EGF-R cDNA (pSRneo EGF-R [31,36]), was used. ΔWS [37], R129C [38], 8YF [31], 1–251 [39] and ΔBox1 [40] EPO-Rs subcloned in pXM were employed.

Retroviral vectors of PTP1B–GST subcloned between *pacI* and *EcoRI* of pMOWS–GFP (where GFP stands for green fluorescent protein) were transiently transfected in Phoenix-Eco cells by calcium phosphate precipitation as described in [41]. Transducing supernatants were applied to introduce the GST–PTP1B in pMOWS or pMOWS–GFP cDNAs into the IL-3-dependent pro-B cell line Ba/F3 stably expressing wt (wild-type) EPO-R cDNA in pXM [30]. Pools expressing GST–PTP1B or GFP were selected in 1.5 µg/ml puromycin (Sigma) 48 h after transduction. The selected cells were maintained in RPMI 1640 supplemented with 10 % (v/v) foetal calf serum and 0.25 unit/ml EPO in the presence of 1.5 µg/ml puromycin.

Stimulation with EPO

This was performed as described in [40].

Western-blot analysis, immunoprecipitation and digestion with endo H

Cells were solubilized in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 10 mM EDTA, 50 mM sodium fluoride, 5 mM iodoacetic acid, 1 mM sodium orthovanadate and a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Immunoprecipitation and Western-blot analysis were performed as described previously [30]. For digestion with endo H, Protein A–Sepharose pellets were boiled for 3 min in 60 µl of 50 mM sodium citrate (pH 5.6) and 1 % (w/v) SDS. The supernatant was divided into two aliquots that were incubated for 1 h at 37 °C in the presence or absence of 0.5 µl of endo H (stock solution, 1 unit/ml) and then separated by SDS/PAGE.

RESULTS

pTyr EPO-R is detected in cells co-expressing wt EPO-R and PTP1BD

To determine whether the EPO-R is a target of PTP1B, we performed 'substrate-trapping' experiments in BOSC-23T cells. Wt EPO-R was transiently expressed in BOSC-23T cells, together

Figure 1 Detection of pTyr EPO-R in cells co-expressing EPO-R and PTP1BD

BOSC-23T cells were co-transfected with cDNAs of wt EPO-R and pMT2 vector, PTP1B (1B) or PTP1BD (1BD) (lanes 1–3 respectively). Lane 4 represents non-transfected cells. (A) Total cell lysates were subjected to Western-blot analysis and probed with anti-PY antibodies (upper panel) or antibodies directed against the cytosolic domain of the EPO-R (lower panel). (B) Immunoprecipitation of EPO-R from cell lysates using antibodies directed against the cytosolic domain of the EPO-R, followed by addition of Protein A–Sepharose. Immunoprecipitates were subjected to Western-blot analysis with anti-PY antibodies, antibodies directed against the cytosolic domain of the EPO-R or anti-PTP1B antibodies (top, middle or bottom panel respectively). Molecular-mass standards in kDa are shown on the right.

with wt PTP1B or PTP1BD. Cell lysates were immunoprecipitated with anti-EPO-R antibodies, and this was followed by Western-blot analysis with anti-PY antibodies (Figure 1B). We reprobbed the same blot with anti-EPO-R antibodies to compare receptor expression levels (Figure 1B, middle panel), and with anti-PTP1B to determine whether PTP1B forms a complex with the EPO-R (Figure 1B, bottom panel). Co-expression of wt EPO-R and PTP1BD (Figure 1B, lane 3) resulted in the accumulation of pTyr EPO-R, whereas pTyr EPO-R could not be detected in cells co-transfected with EPO-R and PTP1B (Figure 1B, lane 2). Figure 1(A) presents the corresponding Western-blot analysis of total cell lysates probed with anti-PY (Figure 1A, upper panel) or anti-EPO-R antibodies (Figure 1A, lower panel). To determine the specificity of the interaction between EPO-R and PTP1B, we examined whether tyrosine-phosphorylated EPO-R accumulates after co-expression of the EPO-R with TCPTP D182A (TCD), the substrate-trapping mutant of TCPTP [33]. Tyrosine-phosphorylated EPO-R was detected in BOSC-23T cells co-transfected with PTP1BD and EPO-R (Figure 2A, lane 4), whereas in the presence of TCD, pTyr EPO-R was barely distinguished (Figure 2A, lane 2). On the other hand, tyrosine-phosphorylated EGF-R was detected in cells expressing substrate-trapping mutant forms of either TCPTP or PTP1B (Figure 2B, lanes 3 and 5), as described previously [33]. Thus, it seems that EPO-R is a preferential substrate of PTP1B. Tyrosine phosphorylation of Δ Box1 EPO-R, an EPO-R lacking residues 256–267 required for JAK2 activation, was barely detected in the presence of PTP1BD. These results may imply that PTP1B dephosphorylates EPO-Rs that were tyrosine-phosphorylated by JAK2.

In vivo interaction between wt EPO-R and PTP1B

To substantiate the above finding of interaction between the EPO-R and PTP1B, we examined complex formation between EPO-R and GST-fusion proteins of PTP1B (GST–PTP1B) and PTP1BD (GST–PTP1BD). BOSC-23T cells were co-transfected with expression plasmids of wt EPO-R and PTP1B or wt EPO-R and PTP1BD. Cell lysates were precipitated with GSH–Sepharose beads at 4 °C for 3 h, eluted in SDS sample buffer and subjected to immunoblot analysis with anti-PY, anti-EPO-R and anti-PTP1B antibodies. As shown in Figure 3, several tyrosine-phosphorylated proteins co-precipitated with GST–PTP1BD in cells that did not express the EPO-R. However, in cells expressing the EPO-R together with PTP1BD, most of the tyrosine phosphorylation occurred on a protein entity that was shown by immunoblot analysis to coincide with the EPO-R. These results indicate that EPO-R and PTP1B form a complex *in vivo* (Figure 3, lane 4).

To determine the region on EPO-R that binds to PTP1B, we examined the *in vivo* binding between GST-fusion proteins of PTP1B or PTP1BD and the following receptors: wt EPO-R, an EPO-R mutant devoid of the extracellular domain in which the cytosolic tyrosine residues are replaced by phenylalanine residues (8YF cyt EPO-R), and an EPO-R mutant devoid of its cytosolic region (EPO-R 1–251). Figure 4 depicts complex formation between PTP1B or PTP1BD and the wt EPO-R (Figure 4A, lanes 2 and 3). 8YF cyt EPO-R (Figure 4A, lanes 5 and 6) bound to PTP1B and PTP1BD, whereas 1–251 EPO-R was not detected in the GSH–Sepharose precipitates (Figure 4A, lanes 8 and 9). It should be noted that the amounts of 1–251 EPO-R in the cell

Figure 2 Inability to detect pTyr EPO-R upon co-expression of EPO-R and TCD

BOSC-23T cells were co-transfected with cDNA of wt EPO-R (**A**), Δ Box1 EPO-R (**A**) or EGF-R (**B**) and cDNAs of pMT2 vector, PTP1B (1B), PTP1BD (1BD), TCPTP (TC) or TCD (lanes 1–5 respectively). Lane 6 represents non-transfected cells. EPO-R or EGF-R was immunoprecipitated from cell lysates using specific antibodies, followed by the addition of Protein A–Sepharose. The immunoprecipitates were subjected to Western-blot analysis with anti-PY antibodies. Immunoblots of the respective blots probed with anti-EPO-R or anti-EGF-R are depicted for comparison. Molecular-mass standards in kDa are shown on the right.

Figure 3 Precipitation of pTyr EPO-R in association with PTP1BD

BOSC-23T cells were transfected with the cDNA of GST-1B or GST-1BD (lanes 1 and 2 respectively) or were co-transfected with wt EPO-R and GST-1B or GST-1BD expression plasmids (lanes 3 and 4 respectively). Complexes were precipitated by agarose-bound GSH. Samples were subjected to Western-blot analysis with anti-PY antibodies. The same blots were then probed with anti-EPO-R antibodies directed against the cytosolic domain of the EPO-R or anti-PTP1B antibodies. Molecular-mass standards in kDa are shown on the right.

Figure 4 Mediation of the interaction between EPO-R and PTP1BD via the cytosolic domain of the EPO-R, in the absence of EPO-R cytosolic tyrosine residues

BOSC-23T cells were co-transfected with the cDNAs of wt EPO-R, 8YF cyt EPO-R or 1–251 EPO-R and the cDNAs of pMT2 vector or PTP1B or PTP1BD. (**A**) PTP1B or PTP1BD was immunoprecipitated from cell lysates using anti-PTP1B antibodies, followed by the addition of Protein A–Sepharose. The immunoprecipitates were subjected to SDS/PAGE and blotted on to nitrocellulose membrane filters, which were probed using anti-EPO-R antibodies directed against the cytosolic domain (lanes 1–6) or against the extracellular domain (lanes 7–10) of the EPO-R. (**B**) Total cell lysates were subjected to Western-blot analysis with anti-EPO-R antibodies directed against the cytosolic domain of the EPO-R (lanes 1–6) or the extracellular domain of EPO-R (lanes 7–10). Molecular-mass standards in kDa are shown on the right.

extracts were higher than those of wt EPO-R or 8YF cyt EPO-R (Figure 4B). The finding that 8YF cyt EPO-R binds to both PTP1B and PTP1BD indicates that this interaction occurs through the cytosolic domain of the EPO-R and that the binding can occur in the absence of tyrosine residues.

Endo H-resistant EPO-R is tyrosine-phosphorylated upon co-transfection with PTP1BD and EPO-R

To determine the intracellular location of tyrosine-phosphorylated EPO-R in the presence of PTP1BD, we examined the susceptibility of pTyr EPO-R to endo H. This was done by endo H treatment of immunoprecipitates of the EPO-R from cells co-expressing wt EPO-R and PTP1BD, followed by immunoblotting with anti-PY antibodies. Immunoblotting with anti-EPO-R antibodies showed that most of the EPO-R molecules were sensitive to endo H and were not tyrosine-phosphorylated (Figure 5A, lanes 1 and 2). Tyrosine-phosphorylated EPO-Rs, on the other hand, were predominantly resistant to endo H treatment (Figure 5A, lanes 5 and 6). Thus, it seems probable that these endo H-resistant pTyr EPO-Rs are not detected by the anti-EPO-R antibodies in immunoblots, and that this is due both to their low abundance and to the greater potency of the anti-PY antibodies compared with that of the anti-EPO-R antibodies (Figure 5A, lower panel, lanes 5 and 6). To examine whether the endo H-resistant pTyr EPO-R molecules form a complex with PTP1BD, we immunoprecipitated the cell lysates with anti-PTP1B antibodies and subjected the immunoprecipitates to endo H digestion, followed by Western-blot analysis with anti-PY antibodies and anti-EPO-R antibodies.

Figure 5 Endo H susceptibility of pTyr EPO-R

BOSC-23T cells were co-transfected with wt EPO-R and cDNAs of pMT2 vector, PTP1B or PTP1BD. EPO-R (**A**) or PTP1B (**B**) was immunoprecipitated from cell lysates using specific antibodies, followed by the addition of Protein A–Sepharose. The Protein A–Sepharose pellets were subjected to endo H digestion, followed by Western-blot analysis with anti-PY. The membrane filters were stripped and then probed with anti-EPO-R cytosolic antibodies (**A**, **B**, lower panels). Membrane filters were probed with anti-PY or anti-EPO-R antibodies. The 75 kDa molecular-mass standard is shown on the right.

This analysis demonstrated that most of the EPO-R molecules that were complexed with PTP1BD displayed sensitivity to endo H (Figure 5B, lower panel, lanes 5 and 6). However, tyrosine phosphorylation was detected mainly on endo H-resistant EPO-R molecules (Figure 5B, upper panel, lanes 5 and 6). Results of this analysis suggest that the EPO-R and PTP1B form a complex in the ER, but that tyrosine phosphorylation which is susceptible to PTP1B dephosphorylation occurs beyond the ER and the *cis* Golgi.

An EPO-R mutant (Δ WS1) that lacks the first WS amino acids of the WSXWS motif is evidently retained in the ER, as shown by its inability to acquire resistance to endo H and to bind EPO on the cell surface and by its immunofluorescence localization [37]. To establish further whether dephosphorylation of the EPO-R by PTP1B (Figure 5) occurs beyond the ER, we examined whether the ER-retained Δ WS1 EPO-R mutant is tyrosine-phosphorylated after its co-expression with PTP1BD. The inability to detect tyrosine-phosphorylated Δ WS1 EPO-R in BOSC-23T cells co-expressing this EPO-R mutant together with PTP1BD (Figure 6) further supports our contention that EPO-Rs that are phosphorylated beyond the ER are dephosphorylated by PTP1B.

After subjecting BOSC-23T cells expressing wt or Δ WS1 EPO-Rs to PV treatment, we found that both tyrosine-phosphorylated wt and tyrosine-phosphorylated Δ WS1 EPO-Rs were detected, as shown previously for Ba/F3 cells expressing these receptors [9]. In BOSC-23T cells expressing wt EPO-R, PV treatment led to the accumulation of endo H-sensitive (>90%) and endo H-resistant pTyr EPO-Rs (Figure 6, lanes 3 and 4). On the other hand, only

Figure 6 Tyrosine phosphorylation of EPO-R mutants in the presence of PTP1BD and/or PV

BOSC-23T cells were transfected with wt or Δ WS EPO-Rs with or without PTP1BD, as indicated. Cells were collected 48 h after transfection and incubated with or without PV (1 mM sodium vanadate and 2 mM hydrogen peroxide) at 37 °C for 10 min. The EPO-Rs precipitated from cell lysates were subjected to endo H digestion, followed by Western-blot analysis with anti-PY, antibodies directed against the cytosolic domain of the EPO-R and anti-PTP1B. Arrowhead and arrows point at endo H sensitive and resistant EPO-R forms respectively.

endo H-sensitive pTyr Δ WS1 EPO-R could be discerned (Figure 6, lanes 9 and 10). After transfecting BOSC-23T cells with wt or Δ WS1 EPO-R together with PTP1BD, we compared the profiles of pTyr EPO-Rs in these cells in the presence or absence of PV. Immunoblot analysis of immunoprecipitated EPO-Rs showed that pTyr wt EPO-Rs from PV-treated cells expressing both EPO-R and PTP1BD were predominantly resistant to endo H treatment, unlike PV-treated cells that were transfected with wt EPO-R only (Figure 6, cf. lanes 5 and 6 with lanes 3 and 4). Notably, after PV treatment, pTyr Δ WS1 EPO-R was sensitive to endo H digestion regardless of whether the EPO-R was expressed without or with PTP1BD (Figure 6, lanes 9–12). Therefore it appears that PTP1B dephosphorylates mature EPO-Rs. Co-immunoprecipitation of PTP1B with both wt and Δ WS EPO-Rs further supports the data presented in Figure 6, which suggest binding of PTP1B to ER-localized EPO-R and dephosphorylation in subsequent compartments of the secretory pathway.

To determine whether PTP1B dephosphorylates ligand-activated EPO-Rs, we subjected BOSC-23T cells co-expressing wt EPO-R and PTP1BD or wt EPO-R only to activation with EPO. As shown in Figure 7, stimulation with EPO increased the amount of pTyr EPO-Rs in EPO-R/PTP1BD-expressing cells in a time-dependent manner: EPO-R tyrosine phosphorylation reached a peak 1 min after the onset of EPO stimulation and returned to basal pTyr levels after 30 min (Figure 7A and lower panel). We therefore conclude that PTP1B participates in the down-regulation of EPO-mediated signal transduction, probably by dephosphorylating cell-surface ligand-activated EPO-Rs. In the absence of PTP1BD, EPO-mediated tyrosine phosphorylation of the EPO-R was not detected (Figure 7B). This might be explained by the relatively small amounts of cell-surface EPO-R and the rapid dephosphorylation of ligand-activated EPO-R.

The involvement of PTP1B in the dephosphorylation of the EPO-R after ligand stimulation was also demonstrated in the pro-B cell line Ba/F3. These cells do not have endogenous EPO-Rs and depend on IL-3 for proliferation. Yet, when transfected with the EPO-R cDNA, they can become dependent on EPO [29]. To examine the role of PTP1B in EPO-R signalling, we generated Ba/F3 cells that co-express the EPO-R and PTP1B in pMOWS or EPO-R and pMOWS–GFP (control). These cells were stimulated with EPO, and pTyr-immunoprecipitated EPO-R was monitored

Figure 7 Tyrosine phosphorylation of ligand-activated EPO-R in the presence of PTP1BD

BOSC-23T cells were co-transfected with expression plasmids of wt EPO-R and PTP1BD (A) or transfected with EPO-R only (B). Cells were collected 48 h after transfection and incubated with EPO (100 units/ml) at 37 °C for the indicated time periods. Immunoprecipitated EPO-Rs were subjected to Western-blot analysis with anti-PY and anti-EPO-R cytosolic domain antibodies. Lower panel: densitometric analysis of pTyr EPO-R in EPO-R/PTP1BD-expressing cells (presented in arbitrary units relative to the amount of total EPO-R) as a function of the time of EPO stimulation is shown.

with respect to the amount of immunoprecipitated EPO-R. Figure 8 demonstrates that tyrosine phosphorylation of the EPO-R in EPO-R- and PTP1B-expressing cells was lower than that observed in cells expressing the EPO-R only. Note that 5 min after stimulation with EPO, tyrosine phosphorylation of the EPO-R in PTP1B-expressing cells decreased, whereas in control cells it remained at peak levels.

DISCUSSION

The major finding of the present study is that PTP1B participates in the down-regulation of the mature endo H-resistant EPO-R. Support for this conclusion came from a series of experiments in which we employed the catalytically inactive PTP1BD. These experiments demonstrated that (i) the EPO-R is tyrosine-phosphorylated after co-expression with PTP1BD (Figures 1 and 2), (ii) the cytosolic domain of the EPO-R interacts with PTP1B and this interaction can occur in the absence of pTyr residues on the EPO-R (Figure 4), (iii) expression of PTP1BD leads to the accumulation of endo H-resistant pTyr EPO-R, but not of tyrosine-phosphorylated ER-retained Δ WS1 EPO-R (Figure 6) and (iv) PTP1BD expression increases ligand-mediated tyrosine phosphorylation of the EPO-R (Figure 7). The lower levels of pTyr EPO-R in EPO-stimulated Ba/F3 cells expressing EPO-R and

Figure 8 Participation of PTP1B in down-regulation of EPO-R signalling

Ba/F3 cells co-expressing wt EPO-R and PTP1B, or wt EPO-R and GFP (control), were incubated with EPO (100 units/ml) at 37 °C for the indicated time periods. Immunoprecipitated EPO-Rs were subjected to Western-blot analysis with anti-PY and anti-EPO-R N-terminal antibodies. Lower panel: densitometric analysis of pTyr EPO-R (presented in arbitrary units relative to the amount of total EPO-R) as a function of the time of EPO stimulation is shown. This Figure represents the results of one representative experiment performed in triplicate.

PTP1B further supports the participation of PTP1B in the down-regulation of EPO-R signalling.

In both transfected and normal haematopoietic cells, most of the newly produced EPO-Rs are retained in the ER [9,10,31,32,42], thus directing attention to intracellular EPO-Rs. Our previous study showed that the ER-retained Δ WS1 EPO-R mutant is tyrosine-phosphorylated when PTPs are inhibited by the potent tyrosine phosphatase inhibitor PV [9], suggesting that protein tyrosine kinases are present and are active on the EPO-R in this microenvironment. In a subsequent study, association of the EPO-R with JAK2 in early compartments of the secretory pathway was shown to enhance the folding of EPO-R and facilitate its surface expression [10]. Association of the EPO-R with JAK2 within the ER environment suggested that the receptor might also form complexes with a 'balancing' phosphatase moiety(s). Recently, JAK2 was implicated in the quantitative modulation of intracellular growth hormone receptor [11], suggesting that such modulation might be a common feature of cytokine receptors. In the present study, we have shown that immature endo H-sensitive EPO-R was the predominant receptor species in the PTP1BD/EPO-R complexes. Unexpectedly, this EPO-R was not tyrosine-phosphorylated (Figure 5), in contrast with the tyrosine-phosphorylated EPO-Rs that precipitated with PTP1BD and were derived from the considerably less abundant species of endo H-resistant EPO-R. The inability to detect tyrosine-phosphorylated Δ WS1 EPO-R and tyrosine-phosphorylated immature EPO-Rs in the presence of PTP1BD (Figure 6) further supports our contention that the mature EPO-R is dephosphorylated by PTP1B.

The EPO-R, while residing in the ER, most probably forms complexes with other PTPs in addition to PTP1B. This is inferred by the predominant accumulation of pTyr endo H-sensitive forms

of wt and Δ WS1 EPO-Rs after general inhibition of PTPs by PV (Figure 6). Mature pTyr EPO-R, which accumulates in EPO-R- and PTP1BD-expressing cells that were treated with PV (Figure 6), could be derived from maturation of pTyr endo H-sensitive EPO-Rs or from cell-surface EPO-Rs. The first possibility is supported by a previous demonstration by our group, in which experiments utilizing pulse-chase analysis showed that pTyr endo H-sensitive EPO-Rs accumulate after PV treatment and mature in the course of the chase [9]. Nevertheless, at least some of the pTyr EPO-R derives from the cell surface, as its amount increases after ligand stimulation (Figure 7). The shift from endo H-sensitive to endo H-resistant pTyr EPO-Rs after stimulation by PV of cells expressing PTP1BD suggests that PTP1B comprises only a partial repertoire of the PTPs that dephosphorylate the EPO-R. In a previous study [9], it was demonstrated that most of the pTyr EPO-Rs in PV-treated Ba/F3 and in mouse foetal liver cells were resistant to endo H, suggesting that the balance between mature and immature pTyr EPO-Rs is probably cell-dependent.

Binding of ligand to cell-surface EPO-R results in the activation of JAK2 and phosphorylation of tyrosine residues in the cytosolic domain of the receptor. Termination of EPO-R signalling is attributed to the cytosolic SH-PTP1 that binds to pTyr⁴²⁹ in the cytosolic domain of EPO-R, leading to dephosphorylation and inactivation of JAK2 [3]. The observed increase in ligand-mediated tyrosine phosphorylation of the EPO-R in the presence of PTP1BD (Figure 7) suggests that PTP1B, which is anchored to the ER, participates in the down-regulation of the ligand-activated cell-surface EPO-R. Further support for this suggestion comes from the finding that trypsin treatment (performed at 4 °C) of cells that were stimulated with EPO resulted in a significant decrease in pTyr EPO-R, whereas the amounts of intracellular EPO-R were unaffected (results not shown). Thus, the ER-localized PTP1B might catalyse EPO-R dephosphorylation at specific well-segregated sites on the ER after ligand activation, similar to its dephosphorylation of ligand-stimulated EGF-R and platelet-derived growth factor receptor tyrosine kinases [25]. The fact that tyrosine phosphorylation of ligand-activated EPO-R decreases in the presence of PTP1BD (Figure 7) is in line with the contribution of other phosphatases to this process. Further support for the involvement of PTP1B in the down-regulation of ligand-activated EPO-R stems from our finding that tyrosine phosphorylation of the EPO-R after EPO stimulation is lower in Ba/F3 cells which express both EPO-R and PTP1B. So far, we were not successful in generating EPO-R-expressing Ba/F3 cells that also express PTP1BD. Recent studies demonstrated enhanced effects of leptin [43] and growth hormone [44] in PTP1B-deficient mice or in the presence of PTP1BD. It has been suggested that PTP1B targets JAK2. Despite the interaction between EPO-R and PTP1B, which we have demonstrated, our studies cannot rule out that the involvement of PTP1B in the down-regulation of EPO-R signalling is mediated via JAK2.

Previous studies have demonstrated that PTP1BD traps the EGF-R in distinct punctate structures within the ER [22]. Immunofluorescence labelling showed that expression of the EPO-R along with PTP1B did not alter the intracellular localization of the EPO-R (results not shown). This might be attributable to the difference in subcellular distribution of the EGF-R and the EPO-R; whereas the former is localized mainly at the cell surface, the latter resides mainly in early compartments of the secretory pathway [31,32].

Since pTyr EPO-R was not detected in cells expressing TCD (Figure 2), our results probably represent a genuine interaction between the EPO-R and PTP1B and not merely the result of overexpression of a catalytically inactive PTP1B and EPO-R in BOSC-23T cells. Additional support for this interaction comes

from the observed binding of wt EPO-R to GST-PTP1B- and GST-PTP1BD-fusion proteins *in vivo* (Figure 3).

The notion that a combination of pTyr residues may be crucial for PTP1B interaction has gained support from studies on the insulin receptor, where Tyr¹¹⁴⁶, Tyr¹¹⁵⁰ and/or Tyr¹¹⁵¹ were shown to be important for complex formation with PTP1B [24]. However, the interaction between EPO-R and PTP1BD can occur in the absence of tyrosine phosphorylation of the EPO-R, as an EPO-R mutant that lacks the extracellular domain (in which all eight cytosolic tyrosine residues were replaced by phenylalanine residues) co-precipitated with PTP1B and PTP1BD (Figure 4). Future studies should address key issues such as the identity of pTyr residue(s) of the EPO-R that are dephosphorylated by PTP1B and the involvement of other phosphatases and kinases in this process.

In conclusion, our study suggests that PTP1B modulates EPO signalling via its cell-surface receptor. Thus, PTP1B may inactivate central proteins that participate in the cytokine-mediated signal transduction pathway, including the cell-surface receptors JAK2 and STAT5.

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REFERENCES

- Constantinescu, S. N., Ghaffari, S. and Lodish, H. F. (1999) The erythropoietin receptor: structure, activation and intracellular signal transduction. *Trends Endocrinol. Metab.* **10**, 18–23
- Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. and Ihle, J. N. (1993) Jak2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell (Cambridge, Mass.)* **74**, 227–236
- Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G. and Lodish, H. F. (1995) Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of Jak2 and termination of proliferative signals. *Cell (Cambridge, Mass.)* **80**, 729–738
- Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K. and Wilson, I. A. (1999) Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science* **283**, 987–990
- Constantinescu, S. N., Huang, L. J., Nam, H. and Lodish, H. F. (2001) The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. *Mol. Cell* **7**, 377–385
- Kubatzky, K. F., Ruan, W., Gurezka, R., Cohen, J., Ketteler, R., Watowich, S. S., Neumann, D., Langosch, D. and Klingmuller, U. (2001) Self assembly of the transmembrane domain promotes signal transduction through the erythropoietin receptor. *Curr. Biol.* **11**, 110–115
- Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell (Cambridge, Mass.)* **78**, 1027–1037
- Posner, B. I., Faure, R., Burgess, J. W., Bevan, A. P., Lachance, D., Zhang Sun, G., Fantus, I. G., Ng, J. B., Hall, D. A., Lum, B. S. et al. (1994) Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. *J. Biol. Chem.* **269**, 4596–4604
- Cohen, J., Altaratz, H., Zick, Y., Klingmuller, U. and Neumann, D. (1997) Phosphorylation of erythropoietin receptors in the endoplasmic reticulum by pervanadate-mediated inhibition of tyrosine phosphatases. *Biochem. J.* **327**, 391–397
- Huang, L. J., Constantinescu, S. N. and Lodish, H. F. (2001) The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. *Mol. Cell* **8**, 1327–1338
- Moulin, S., Bouzinba-Segard, H., Kelly, P. A. and Finidori, J. (2003) Jak2 and proteasome activities control the availability of cell surface growth hormone receptors during ligand exposure. *Cell Signal.* **15**, 47–55
- Frank, S. J. (2002) Receptor dimerization in GH and erythropoietin action – it takes two to tango, but how? *Endocrinology* **143**, 2–10

- 13 Lotti, L. V., Lanfranccone, L., Migliaccio, E., Zompetta, C., Pelicci, G., Salcini, A. E., Falini, B., Pelicci, P. G. and Torrisi, M. R. (1996) Sch proteins are localized on endoplasmic reticulum membranes and are redistributed after tyrosine kinase receptor activation. *Mol. Cell. Biol.* **16**, 1946–1954
- 14 Bauskin, A. R., Alkalay, I. and Ben Neria, Y. (1991) Redox regulation of a protein tyrosine kinase in the endoplasmic reticulum. *Cell* (Cambridge, Mass.) **66**, 685–696
- 15 Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, II, R. L., Cox, A. D. and Philips, M. R. (2002) Ras signalling on the endoplasmic reticulum and the Golgi. *Nat. Cell Biol.* **4**, 343–350
- 16 Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A. and Neel, B. G. (1992) The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell* (Cambridge, Mass.) **68**, 545–560
- 17 Cheng, A., Bal, G. S., Kennedy, B. P. and Tremblay, M. L. (2001) Attenuation of adhesion-dependent signaling and cell spreading in transformed fibroblasts lacking protein tyrosine phosphatase-1B. *J. Biol. Chem.* **276**, 25848–25855
- 18 Rhee, J., Lilien, J. and Balsamo, J. (2001) Essential tyrosine residues for interaction of the non-receptor protein-tyrosine phosphatase PTP1B with N-cadherin. *J. Biol. Chem.* **276**, 6640–6644
- 19 Myers, M. P., Andersen, J. N., Cheng, A., Tremblay, M. L., Horvath, C. M., Parisien, J. P., Salmeen, A., Barford, D. and Tonks, N. K. (2001) Tyk2 and jak2 are substrates of protein-tyrosine phosphatase 1B. *J. Biol. Chem.* **276**, 47771–47774
- 20 Espanel, X., Walchli, S., Gobert, R. P., El Alama, M., Curchod, M. L., Gullu-Isler, N. and van Huijsduijnen, R. H. (2001) Pulling strings below the surface: hormone receptor signaling through inhibition of protein tyrosine phosphatases. *Endocrine* **15**, 19–28
- 21 Aoki, N. and Matsuda, T. (2000) A cytosolic protein tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. *J. Biol. Chem.* **275**, 39718–39725
- 22 Flint, A. J., Tiganis, T., Barford, D. and Tonks, N. K. (1997) Development of 'substrate-trapping' mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1680–1685
- 23 Liu, F. and Chernoff, J. (1997) Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. *Biochem. J.* **327**, 139–145
- 24 Bandyopadhyay, D., Kusari, A., Kenner, K. A., Liu, F., Chernoff, J., Gustafson, T. A. and Kusari, J. (1997) Protein-tyrosine phosphatase 1B complexes with the insulin receptor *in vivo* and is tyrosine-phosphorylated in the presence of insulin. *J. Biol. Chem.* **272**, 1639–1645
- 25 Haj, F. G., Verveer, P. J., Squire, A., Neel, B. G. and Bastiaens, P. I. (2002) Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. *Science* **295**, 1708–1711
- 26 Haj, F. G., Markova, B., Klamann, L. D., Bohmer, F. D. and Neel, B. G. (2003) Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatase-1B. *J. Biol. Chem.* **278**, 739–744
- 27 Lavoie, C., Chevet, E., Roy, L., Tonks, N. K., Fazel, A., Posner, B. I., Paiement, J. and Bergeron, J. J. (2000) Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13637–13642
- 28 Gelderloos, J. A. and Anderson, S. M. (1996) Over-expression of protein tyrosine phosphatase 1 (PTP1) alters IL-3-dependent growth and tyrosine phosphorylation. *Oncogene* **13**, 2367–2378
- 29 Li, J. P., D'Andrea, A. D., Lodish, H. F. and Baltimore, D. (1990) Activation of cell growth by binding of friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. *Nature* (London) **343**, 762–764
- 30 Neumann, D., Wikstrom, L., Watowich, S. S. and Lodish, H. F. (1993) Intermediates in degradation of the erythropoietin receptor accumulate and are degraded in lysosomes. *J. Biol. Chem.* **268**, 13639–13649
- 31 Supino-Rosin, L., Yoshimura, A., Altaratz, H. and Neumann, D. (1999) A cytosolic domain of the erythropoietin receptor contributes to endoplasmic reticulum-associated degradation. *Eur. J. Biochem.* **263**, 410–419
- 32 Yoshimura, A., D'Andrea, A. D. and Lodish, H. F. (1990) Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4139–4143
- 33 Tiganis, T., Bennett, A. M., Ravichandran, K. S. and Tonks, N. K. (1998) Epidermal growth factor receptor and the adaptor protein p52shc are specific substrates of T-cell protein tyrosine phosphatase. *Mol. Cell. Biol.* **18**, 1622–1634
- 34 Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8392–8396
- 35 Chen, C. A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6**, 632–638
- 36 Collins, M. K., Downward, J., Miyajima, A., Maruyama, K., Arai, K. and Mulligan, R. C. (1988) Transfer of functional EGF receptors to an IL3-dependent cell line. *J. Cell Physiol.* **137**, 293–298
- 37 Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y. and Lodish, H. F. (1992) Mutations in the trp-ser-x-trp-ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. *J. Biol. Chem.* **267**, 11619–11625
- 38 Yoshimura, A., Longmore, G. and Lodish, H. F. (1990) Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. *Nature* (London) **348**, 647–649
- 39 Levin, I., Cohen, J., Supino-Rosin, L., Yoshimura, A., Watowich, S. S. and Neumann, D. (1998) Identification of a cytoplasmic motif in the erythropoietin receptor required for receptor internalization. *FEBS Lett.* **427**, 164–170
- 40 Flint-Ashtamker, G., Eisen-Lev, R., Cohen, J., Jun-Shen Huang, L. and Neumann, D. (2002) Amino acid residues 268–276 of the erythropoietin receptor contain an endocytosis motif and are required for erythropoietin-mediated proliferation. *FEBS Lett.* **518**, 189–194
- 41 Ketteler, R., Heinrich, A. C., Offe, J. K., Becker, V., Cohen, J., Neumann, D. and Klingmuller, U. (2002) A functional green fluorescent protein–erythropoietin receptor despite physical separation of Jak2 binding site and tyrosine residues. *J. Biol. Chem.* **277**, 26547–26552
- 42 Hilton, D. J., Watowich, S. S., Murray, P. J. and Lodish, H. F. (1995) Increased cell surface expression and enhanced folding in the endoplasmic reticulum of a mutant erythropoietin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 190–194
- 43 Zabolotny, J. M., Bence-Hanulec, K. K., Stricker-Krongrad, A., Haj, F., Wang, Y., Minokoshi, Y., Kim, Y. B., Elmquist, J. K., Tartaglia, L. A., Kahn, B. B. et al. (2002) PTP1B regulates leptin signal transduction *in vivo*. *Dev. Cell* **2**, 489–495
- 44 Gu, F., Dube, N., Kim, J. W., Cheng, A., Ibarra-Sanchez Mde, J., Tremblay, M. L. and Boisclair, Y. R. (2003) Protein tyrosine phosphatase 1B attenuates growth hormone-mediated JAK2-STAT signaling. *Mol. Cell. Biol.* **23**, 3753–3762

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