

# Phosphorylation of erythropoietin receptors in the endoplasmic reticulum by pervanadate-mediated inhibition of tyrosine phosphatases

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Erythropoietin (EPO) is the major hormone regulating the proliferation of erythroid precursors and their differentiation into erythrocytes. Ligand binding to the erythropoietin receptor (EPO-R), a member of the cytokine receptor family, triggers Tyr phosphorylation of the surface form of the receptor, presumably mediated by the Janus kinase (JAK) 2. To study whether non-surface EPO-R can be phosphorylated, Ba/F3 cells stably transfected with EPO-R were treated with pervanadate (PV), which is widely used as a potent tool to inhibit cellular protein Tyr phosphatases, thus resulting in enhanced Tyr phosphorylation of cellular proteins. PV treatment caused the EPO-R to undergo Tyr phosphorylation in a time-dependent and dose-dependent manner. PV-mediated Tyr phosphorylation of EPO-R occurred at several intracellular sites including the endoplasmic reticulum (ER), because both endoglycosidase H (endo H)-resistant EPO-R and the ER-retained EPO-R mutant ( $\Delta$ WS1 EPO-R) were Tyr phosphorylated in response to PV.

Moreover, in metabolic labelling experiments, endo H-sensitive EPO-R was also phosphorylated. The phosphorylated fraction accounted for only 30–50% of the newly synthesized EPO-R, the fraction that normally exits from the ER. Tyr phosphorylation could not be detected on proteolytic fragments of the EPO-R, suggesting that this is a highly regulated process. Unlike the wild-type (wt) EPO-R, which was phosphorylated both on EPO binding and after inhibition of Tyr phosphatases by PV treatment, an EPO-R mutant (W282R EPO-R) that does not activate JAK2 was phosphorylated after PV treatment but not by EPO binding. Both EPO-R and JAK2 were phosphorylated with similar kinetics by PV treatment, suggesting that JAK2, as well as protein Tyr kinases different from JAK2, might mediate PV-dependent EPO-R phosphorylation. Furthermore the Tyr-phosphorylated ER-retained EPO-R mutant  $\Delta$ WS1 co-immunoprecipitated with JAK2 kinase, indicating that the EPO-R might interact with JAK2 while in the ER.

## INTRODUCTION

Erythropoietin (EPO) is the prime regulator that promotes the viability, proliferation and differentiation of mammalian erythroid progenitor cells via its specific cell-surface receptor. The erythropoietin receptor (EPO-R) is a member of the cytokine receptor superfamily characterized by, among other features, the lack of intrinsic kinase activity [1–4]. On ligand binding, cell-surface EPO-R undergoes Tyr phosphorylation [5,6] presumably mediated by the Janus kinase (JAK) 2 [7,8]. The Tyr-phosphorylated EPO-R then serves as a docking site for other cellular proteins, including cellular kinases, which in concert with JAK2 might mediate the cellular response to EPO.

One way to study EPO-mediated phosphorylation of cellular proteins is by inhibiting cellular phosphatases. This could be achieved for example by incubation of cells with pervanadate (PV) compounds, which are the most potent inhibitors of cellular protein Tyr phosphatases (PTPs) yet described [9]. PV compounds are known oxidants; their mode of action probably involves oxidation of a cysteine residue present within a well-conserved sequence in the active site of all PTPs [10]. As a result the activity of PTPs is effectively inhibited. Therefore treatment of cells with PV perturbs the basal equilibrium of Tyr phosphorylation/dephosphorylation, thus resulting in elevated protein Tyr phos-

phorylation [11–14]. In the insulin receptor PV was found to mimic the lipogenic effect of insulin via activation of the insulin receptor Tyr kinase [15], resulting in the phosphorylation of cellular substrates [11,12]. PV was also shown to mimic the events mediated by activation of the T-cell antigen receptor [16,17] and to activate intracellular signalling pathways mediated by the interleukin (IL) 2 receptor [18], to activate STAT1 [19] and to activate the mitogen-activated protein kinase pathway [20]. A convenient cellular system to study the EPO-R is the IL-3-dependent pro-B-cell line Ba/F3. When these cells are transfected with the EPO-R cDNA, the cells can grow in the presence of EPO as well as IL-3 [21]. In these transfected cells, most EPO-R is intracellular, with only about 1500 receptor molecules present on each cell surface [22,23]. Hence this experimental system might be useful for studying both cell-surface and intracellular EPO-R.

In this study we employed PV as a tool to inhibit PTPs involved in dephosphorylation of the EPO-R. Because PV can easily penetrate the cell membrane [11], it serves as a unique tool to activate both the cell-surface and intracellular forms of the EPO-R as opposed to stimulation with EPO, which only allows the analysis of cell-surface forms of the EPO-R. We show that the endoglycosidase H (endo H)-resistant form of the EPO-R is Tyr phosphorylated on stimulation with PV, similarly to EPO-

Abbreviations used: EPO, erythropoietin; EPO-R, erythropoietin receptor; IL, interleukin; JAK2, Janus kinase 2; endo H, endoglycosidase H; ER, endoplasmic reticulum; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; P-Tyr, phosphotyrosine; PV, pervanadate; wt, wild-type.

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induced Tyr phosphorylation of the EPO-R. In pulse-chase experiments we demonstrate that the endo H-sensitive form of the EPO-R is also phosphorylated. Furthermore an endoplasmic reticulum (ER)-retained EPO-R mutant  $\Delta$ WS1 [24] was Tyr phosphorylated on stimulation with PV and co-immunoprecipitated with JAK2 kinase, thus indicating that EPO-R phosphorylation might also occur in the ER and that the EPO-R might assemble with JAK2 while in the ER.

## EXPERIMENTAL

### Materials

Hydrogen peroxide and sodium orthovanadate were obtained from Sigma. Purified recombinant human EPO was a gift from Kirin, Japan. All other materials were obtained from sources previously listed [23].

### Antibodies

Rabbit antibodies directed against a glutathione S-transferase fusion protein containing the intracellular domain of the murine EPO-R or against a glutathione S-transferase fusion protein containing the extracellular domain of the murine EPO-R [8] were used at a dilution of 1:250 for immunoprecipitations and at a dilution of 1:1000 for immunoblotting. A monoclonal antibody against phosphotyrosine (P-Tyr), PY20 (Transduction Laboratories), was employed. Rabbit anti-(murine JAK2) antibodies from Upstate Biotechnology and from Santa Cruz Biotechnology were used at 1:1000 for immunoprecipitation and for immunoblotting respectively.

### Cells and plasmids

Ba/F3 cell lines stably expressing the EPO-R cDNA in pXM [23] were maintained in RPMI medium supplemented with 10% (v/v) fetal calf serum and 0.25 unit/ml EPO. Ba/F3 cell lines stably expressing  $\Delta$ WS1 EPO-R were described previously [24]. Ba/F3 cells stably expressing W282R EPO-R and the cDNA of 1–257 EPO-R [25] were a gift from Dr. S. Watowich (Houston, TX, U.S.A.).

### PV preparation

PV was prepared by mixing  $H_2O_2$  and vanadate into RPMI medium containing 20 mM Hepes, pH 7.5, to final concentrations of 2 and 1 mM respectively. Catalase (Sigma; 2100 units/ml) was added for 10 min to remove excess peroxide. Cells were collected by centrifugation and washed once in RPMI medium with no additives. Cells were then resuspended in either RPMI medium (control) or RPMI containing PV. Subsequently cells were centrifuged and washed once in PBS. In metabolic labelling experiments, a 10-fold concentrated PV solution was added during the chase period. For experiments in which JAK2 was immunoprecipitated, cells were starved for 5 h in the presence of 100 mg/ml BSA (tissue culture grade; Sigma), before stimulation with PV or EPO.

### Metabolic labelling, immunoprecipitation, and digestion with endo H

Ba/F3 cells expressing the EPO-R ( $2 \times 10^6$  cells for each time point) were labelled with [ $^{35}$ S]Cys-Met as described previously [23]. Cells were then chased in the presence of unlabelled amino acids for the indicated periods of time. Solubilization of the cells and immunoprecipitation were performed as described previously

[23]. For co-immunoprecipitating EPO-R with JAK2, cell lysis was performed in 1% (w/v) digitonin as described [3]. For digestion with endo H, the Protein A-Sepharose pellets were boiled for 3 min in 60  $\mu$ l of 50 mM sodium citrate (pH 5.6)/1% (w/v) SDS. The supernatant was divided into two equal aliquots that were incubated overnight at 37 °C in the presence or absence of 2  $\mu$ l of endo H (stock solution 1 unit/ml), before separation on SDS/10% (w/v) polyacrylamide gels.

### Western blot analysis

This was performed as described previously [23]. Acrylamide gels were made from either 7.5% or 10% (w/v) acrylamide with low bisacrylamide, as described [8].

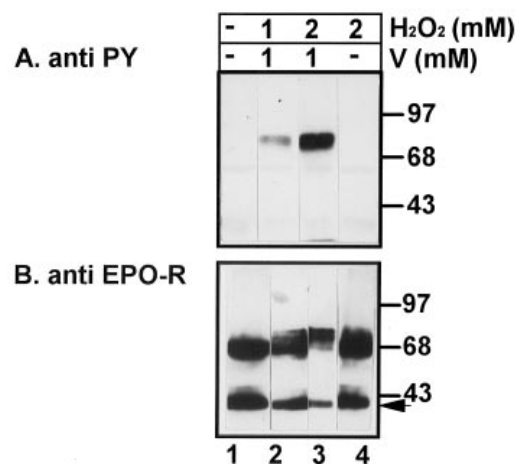
### Preparation of fetal liver cells

Fetal livers were excised from 15-day-old C57Bl/6 mice embryos. Cells were passed through a mesh filter and washed twice in RPMI medium with no additives before treatment with PV or EPO (100 units/ml).

## RESULTS

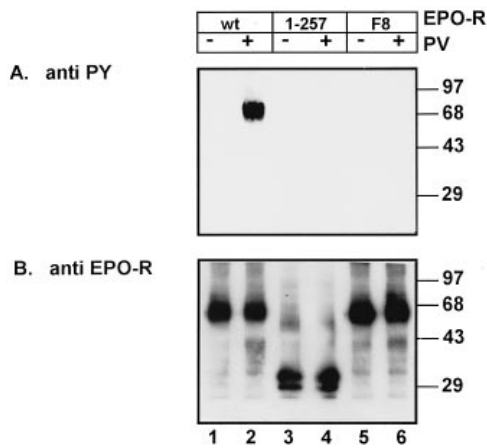
### Stimulation with PV triggers Tyr phosphorylation of the full-length EPO-R but not of its 39 kDa proteolytic fragment

Ba/F3 cells expressing wt EPO-R were treated for 10 min with vanadate and  $H_2O_2$  separately and in combination, to generate PV compounds. Cell lysates were then immunoprecipitated with antibodies directed to the cytoplasmic domain of the EPO-R, separated by SDS/PAGE [10% (w/v) gel], and blots of the gels were probed with anti-(P-Tyr) antibodies (Figure 1A). After stripping of the antibody, the blot was reacted with antibodies against the cytoplasmic domain of the EPO-R (Figure 1B). Whereas  $H_2O_2$  (Figure 1A, lane 4) when added alone had no



**Figure 1** Treatment with PV results in Tyr phosphorylation of the EPO-R

Ba/F3 cells expressing the EPO-R ( $2 \times 10^6$  cells per experimental point) were incubated for 10 min at 37 °C with  $H_2O_2$  and  $Na_3VO_4$  (V) at the concentrations indicated. Cells were then washed and lysed on ice. The lysates were immunoprecipitated with anti-(EPO-R) antibodies directed against the cytoplasmic region of the receptor. The immunoprecipitates were resolved by SDS/PAGE [10% (w/v) gel] and transferred to nitrocellulose membrane filter. The blot was probed with monoclonal anti-(P-Tyr) (anti-PY) antibodies (A) and after stripping of the membrane, with antibodies directed against the cytoplasmic domain of the EPO-R (B), followed by secondary antibody, and developed using the enhanced chemiluminescence method. The positions of molecular mass markers (in kDa) are indicated at the right.



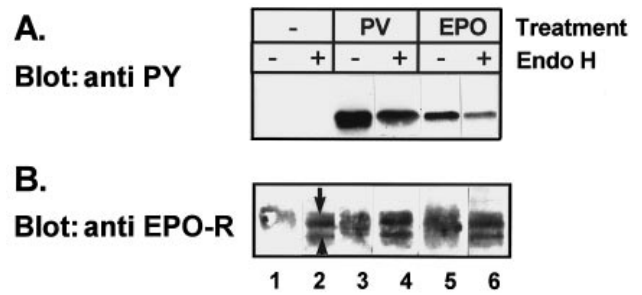
**Figure 2** EPO-R is Tyr phosphorylated in transfected COS cells treated with PV

COS 7 cells were transfected with EPO-R cDNA or a truncated EPO-R (1–257) cDNA, or a cDNA of an EPO-R in which all eight Tyr residues were replaced with Phe (F8). Transfected COS 7 cells were treated with PV for 10 min at 37 °C, washed and solubilized on ice in 500  $\mu$ l of PBS containing 1% (v/v) Triton X-100, 0.5% deoxycholate, 5 mM EDTA, phosphatase inhibitors and protease inhibitors. EPO-R was immunoprecipitated with anti-(EPO-R) antibodies directed against the extracellular domain of the EPO-R, followed by Protein A–Sepharose treatment. The immunoprecipitates were then subjected to SDS/PAGE and blotted on a nitrocellulose membrane filter, which was reacted with anti-(P-Tyr) (anti PY) antibodies (A) or antibodies directed against the extracellular domain of the EPO-R (B). The positions of molecular mass markers (in kDa) are indicated at the right.

effect, treatment with PV resulted in enhanced Tyr phosphorylation of the EPO-R (Figure 1A, lanes 2 and 3), as demonstrated by its reactivity with anti-(P-Tyr) antibodies. After treatment with PV there was a decrease in the mobility of the EPO-R, as detected by anti-(EPO-R) antibodies. The mobility shift of EPO-R could be attributed to its Tyr phosphorylation, as demonstrated by the reactivity of the more slowly migrating EPO-R band with anti-(P-Tyr) antibodies (Figure 1A, lanes 2 and 3). The migration of the 39 kDa EPO-R proteolytic fragment [23] remained similar in PV-treated cells and in control cells (Figure 1B, indicated by arrow). The 39 kDa fragment was recognized by the anti-(EPO-R) antibodies, but not by anti-(P-Tyr) antibodies, indicating that it is not Tyr phosphorylated. From this analysis we determined the optimal concentrations of 1 mM vanadate and 2 mM H<sub>2</sub>O<sub>2</sub> for subsequent experiments.

#### EPO-R is Tyr phosphorylated in transfected COS cells treated with PV

To verify that Tyr-phosphorylated EPO-R and not an associated Tyr-phosphorylated protein reacted with anti-(P-Tyr) antibody, COS 7 cells were transfected with the cDNA of a full-length EPO-R or a truncated EPO-R (1–257), or an EPO-R in which all eight Tyr residues were replaced with Phe (F8) [26]. The results of this experiment demonstrated that although the wt EPO-R and the mutant EPO-Rs were expressed to comparable levels in the COS cells (Figure 2B), only the wt EPO-R was Tyr phosphorylated after treatment with PV (Figure 2A, lane 2). Mutant EPO-Rs lacking either the cytoplasmic region of the receptor or all Tyr residues in the cytoplasmic domain were not phosphorylated (Figure 2A, lanes 3–6). These findings confirmed that the Tyr-phosphorylated protein precipitated by anti-(EPO-R) antibodies was indeed genuine EPO-R.



**Figure 3** Treatment with PV results in Tyr phosphorylation of fetal liver EPO-R

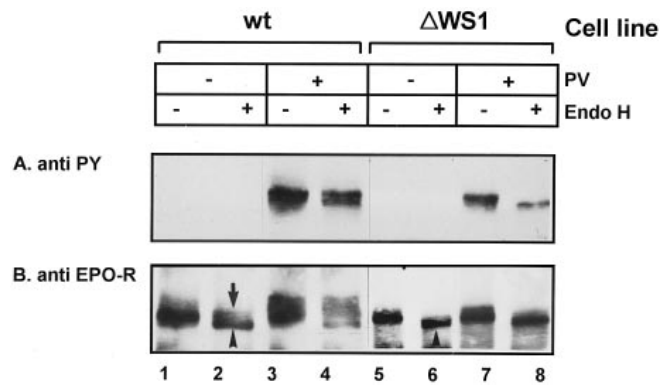
C57Bl/6 mouse fetal liver cells (15 days) were resuspended in PBS (one fetal liver per experimental point) and passed three times through a mesh filter. Cells were resuspended in RPMI medium containing 20 mM Hepes and 10% (v/v) fetal calf serum. Cells were then treated with PV for 10 min at 37 °C, or EPO (100 units/ml) for 5 min at 37 °C or incubated in medium with no additives for 10 min at 37 °C. After the incubation, cells were washed once in PBS and solubilized on ice in 500  $\mu$ l of PBS containing 1% (v/v) Triton X-100, 0.5% deoxycholate, 5 mM EDTA, phosphatase inhibitors and protease inhibitors. EPO-R was immunoprecipitated with anti-(EPO-R) antibodies directed against the intracellular domain of the EPO-R, followed by Protein A–Sepharose treatment. The immunoprecipitates were divided into two aliquots that were incubated overnight in the presence (+) or the absence (–) of endo H. Samples were then resolved by SDS/PAGE [7.5% (w/v) gel] and blotted on nitrocellulose membrane filters, which were reacted with anti-(P-Tyr) (anti PY) antibodies (A) or anti-(EPO-R) antibodies (B). The arrow and arrowhead correspond to endo H-resistant and endo H-sensitive EPO-R respectively.

#### PV augments Tyr phosphorylation of the EPO-R endogenously expressed in fetal liver cells

We next addressed the question of whether PV-induced Tyr phosphorylation of EPO-R could also occur in primary erythroid progenitor cells. Mouse fetal liver cells (15 days) were treated at 37 °C for 10 min with PV, or with EPO (100 units/ml) for 5 min at 37 °C, or with no additives for 10 min at 37 °C. The EPO-R was immunoprecipitated from the lysates with anti-(EPO-R) antibodies directed against the intracellular domain of the EPO-R, followed by Protein A–Sepharose treatment and endo H digestion. Western blot analysis with anti-(P-Tyr) antibodies demonstrated that EPO-R expressed in fetal liver cells was Tyr phosphorylated after treatment with PV (Figure 3A, lanes 3 and 4) similarly to EPO-mediated Tyr phosphorylation of the EPO-R (Figure 3A, lanes 5 and 6). Tyr phosphorylation of the EPO-R was more prominent on treatment with PV than with EPO stimulation, reflecting the potency of treatment with PV. Although endo H-sensitive forms of the EPO-R expressed in fetal liver cells comprise about half of the fetal liver EPO-R molecules (Figure 3B) [27], both PV- and EPO-mediated Tyr-phosphorylated EPO-R were endo H-resistant (Figure 3A, lanes 3–6). These endo H-resistant Tyr-phosphorylated EPO-R bands were sensitive to N-glycanase (results not shown). Thus PV-induced phosphorylation of EPO-R occurs also in cells endogenously expressing the EPO-R, and, in this system, receptor species that are Tyr phosphorylated on treatment with PV resemble those that were Tyr phosphorylated by EPO stimulation to the cells. Because these Tyr-phosphorylated EPO-Rs are endo H-resistant, they are probably located distal to the *cis*-Golgi.

#### An ER-retained EPO-R mutant is Tyr phosphorylated

Although our previous experiments indicated that the Tyr-phosphorylated EPO-Rs are endo H-resistant, we asked whether



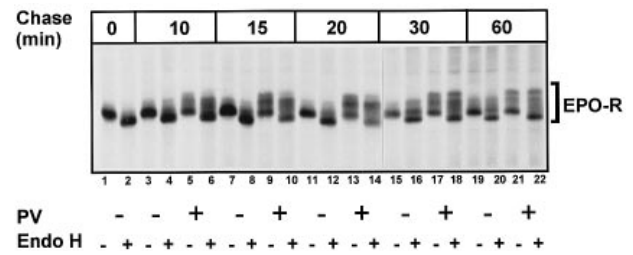
**Figure 4** ER-retained EPO-R mutant is Tyr phosphorylated on treatment with PV

Ba/F3 cells expressing wt EPO-R or  $\Delta$ WS1 EPO-R were incubated for 10 min at 37 °C in the presence or absence of PV. Cells were then washed and lysed on ice. The lysates were immunoprecipitated with anti-(EPO-R) antibodies directed against the cytoplasmic domain of the EPO-R, followed by Protein A–Sepharose treatment. The immunoprecipitates were incubated in the presence or absence of endo H for 14 h at 37 °C. Immunoprecipitates were resolved by SDS/PAGE [10% (w/v) gel] followed by transfer to nitrocellulose filter. The blot was probed with monoclonal anti-(P-Tyr) (anti PY) antibodies (A) or anti-(EPO-R) antibodies (B). The arrowhead and arrow indicate the endo H-sensitive and endo H-resistant EPO-R respectively.

Tyr phosphorylation of EPO-R could also take place intracellularly in the ER. An EPO-R mutant designated  $\Delta$ WS1 was previously shown to be retained in the ER, as determined by its inability to bind EPO on the cell surface, by its inability to acquire resistance to endo H and by its localization in the ER by immunofluorescence [24]. Ba/F3 cells expressing  $\Delta$ WS1 EPO-R and Ba/F3 cells expressing wt EPO-R were exposed to treatment with PV for 10 min. Subsequently cell extracts were immunoprecipitated with anti-(EPO-R) antibodies directed against the cytoplasmic domain of the EPO-R and were Western blotted with anti-(P-Tyr) antibodies. Figure 4 demonstrates that  $\Delta$ WS1 EPO-R was Tyr phosphorylated after treatment with PV (Figure 4A, lanes 7 and 8). Moreover, Tyr-phosphorylated  $\Delta$ WS1 EPO-R was endo H-sensitive (Figure 4A, lanes 7 and 8), indicating that it was phosphorylated in the ER and remained there. In contrast, Tyr-phosphorylated wt EPO-R molecules were mostly endo H-resistant (Figure 4A, lanes 3 and 4). When the same blot was reprobed with anti-(EPO-R) antibodies the  $\Delta$ WS1 mutant was almost exclusively endo H-sensitive (Figure 4B, lanes 6 and 8, indicated by an arrowhead) [24], whereas both endo H-sensitive and endo H-resistant wt EPO-R molecules were observed (Figure 4B, lane 2, arrowhead and arrow respectively). Thus Tyr phosphorylation of the ER-retained EPO-R mutant  $\Delta$ WS1 suggests that Tyr phosphorylation of EPO-R might occur in the ER. The fact that PV-mediated Tyr phosphorylation of the wt EPO-R occurred mainly on its endo H-resistant form suggests that Tyr phosphorylation of the wt EPO-R in the ER might be transient.

#### Wild-type EPO-R is initially phosphorylated in the ER

To test further whether wt EPO-R can be phosphorylated in the ER, metabolic labelling experiments were performed. Ba/F3 cells expressing wt EPO-R were labelled for 30 min with [<sup>35</sup>S]Cys-Met and chased in medium containing unlabelled cysteine and methionine for up to 1 h. PV was added for the last 10 min of each chase. EPO-R was immunoprecipitated from the lysates with anti-(EPO-R) antibodies directed against the intracellular



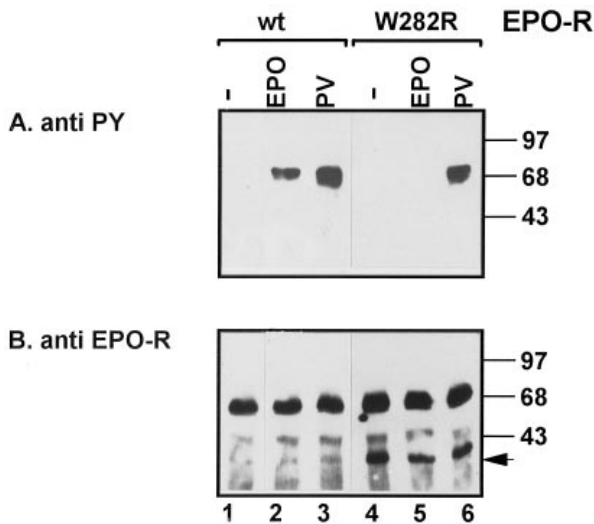
**Figure 5** PV-induced EPO-R phosphorylation occurs before exit of the EPO-R from the ER

Ba/F3 cells expressing the EPO-R were pulse-labelled with [<sup>35</sup>S]Cys-Met for 30 min and then chased for the indicated periods in medium with either no addition or in the presence of PV for the last 10 min of the chase. Detergent extracts of the cells were prepared in PBS containing 1% (w/v) Triton X-100, 5 mM EDTA, 0.5% deoxycholate, protease inhibitors and phosphatase inhibitors. The cell extracts were immunoprecipitated with an antibody directed against the cytoplasmic domain of the EPO-R, and the immunoprecipitates were incubated at 37 °C in the presence or absence of endo H, as indicated. Samples were subjected to SDS/PAGE [10% (w/v) gel] followed by fluorography and autoradiography.

domain of the EPO-R. After the pulse, most of the EPO-R molecules were endo H-sensitive, as previously demonstrated (Figure 5, lanes 1 and 2) [23]. After 10 min of chase, EPO-R was still largely endo H-sensitive. When PV was added during the 10 min of the chase, EPO-R species that were more slowly migrating could be detected. These more slowly migrating EPO-Rs were phosphorylated as demonstrated by their sensitivity to alkaline phosphatase (results not shown). These phosphorylated EPO-R species displayed sensitivity to endo H (Figure 5; compare lanes 3 and 4 with lanes 5 and 6). At 15 min of chase the EPO-R remained mainly endo H-sensitive. The addition of PV for the last 10 min of this chase again resulted in the appearance of more slowly migrating EPO-R species. These EPO-R molecules also displayed sensitivity to endo H, suggesting that PV-induced EPO-R phosphorylation might occur in the ER. After 30 and 60 min of chase in the presence of PV (for the last 10 min), Tyr-phosphorylated EPO-R became mainly endo H-resistant, similar to control, non-phosphorylated, EPO-R (Figure 5; compare lanes 17, 18, 21 and 22 with lanes 15, 16, 19 and 20). Taken together, these results suggest that PV-induced Tyr phosphorylation of wt EPO-R might occur before the exit of the receptor from the ER. PV-mediated phosphorylation of the EPO-R also takes place in later compartments of the secretory pathway, as demonstrated by the phosphorylation of endo H-resistant EPO-R molecules at the longer periods of the chase.

#### W282R EPO-R is Tyr phosphorylated after treatment with PV

JAK2 is probably the first kinase to be activated after binding of EPO to its cell-surface receptor and presumably phosphorylates the EPO-R [7,8]. The EPO-R mutant W282R binds EPO but does not activate JAK2 [28]. To address whether PV-mediated phosphorylation of EPO-R could bypass EPO-mediated activation of JAK2, we exposed Ba/F3 cells expressing W282R EPO-R to PV. Whereas wt EPO-R was phosphorylated on Tyr residues after stimulation with EPO, W282R EPO-R was not Tyr phosphorylated after stimulation with EPO (Figure 6A, lanes 2 and 5 respectively) [28]. In contrast, after treatment of the cells with PV, both wt EPO-R and W282R EPO-R were Tyr phosphorylated (Figure 6A; compare lane 6 with lanes 5 and 4). Note that the 39 kDa fragment was generated from both wt EPO-R and W282R EPO-R (Figure 6B, arrow), indicating that its formation does not require the phosphorylation of EPO-R by



**Figure 6** W282R EPO-R is Tyr phosphorylated by PV

Ba/F3 cells stably expressing W282R EPO-R or wt EPO-R ( $5 \times 10^6$  cells per experimental point) were incubated at 37 °C with PV or with medium only, both for 10 min at 37 °C, or with 100 units/ml EPO for 5 min. The cells were then washed and lysed on ice. EPO-R was immunoprecipitated with anti-(EPO-R) antibodies directed against the cytoplasmic domain of the receptor and samples were processed for Western blot analysis as described. The blot was initially probed with anti-(P-Tyr) (anti PY) antibodies (A); after stripping, it was probed with anti-(EPO-R) antibodies directed against the cytoplasmic domain of the receptor (B). The positions of molecular mass markers (in kDa) are indicated at the right.

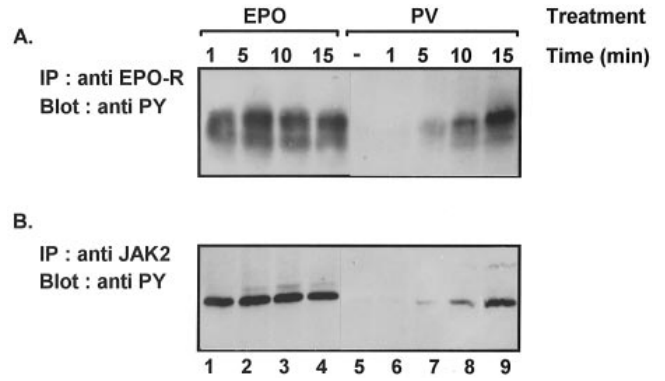
JAK2. These results suggest that PV might activate JAK2, bypassing ligand stimulation, thereby leading to the Tyr phosphorylation of W282R EPO-R. Alternatively, other kinases in addition to JAK2 might be involved in EPO-R phosphorylation.

**JAK2 is phosphorylated by treatment with PV**

To test whether PV activates JAK2 by an EPO-independent pathway, the following experiment was performed. Ba/F3 cells expressing wt EPO-R were treated with either PV or EPO for periods ranging between 1 and 15 min. Lysates of treated cells were immunoprecipitated with either anti-(EPO-R) antibodies or anti-JAK2 antibodies and the blots were probed with anti-(P-Tyr) antibodies. EPO stimulation resulted in Tyr phosphorylation of EPO-R (Figure 7A) and of JAK2 (Figure 7B), which peaked at 5 min of EPO stimulation (Figure 7A, lane 2), as previously shown [3,8]. In contrast, weak Tyr phosphorylation of EPO-R could be observed after 5 min of exposure of the cells to PV, which increased after 10 and 15 min of exposure to PV. Thus the phosphorylation of EPO-R as well as of JAK2 occurred in cells treated either with EPO or with PV. PV-mediated Tyr phosphorylation of EPO-R as well as of JAK2 occurred with similar kinetics.

**$\Delta$ WS1 EPO-R co-immunoprecipitates with JAK2**

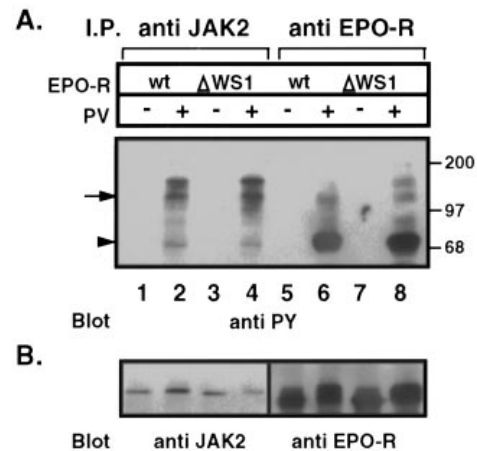
We have shown that intracellular EPO-R can undergo Tyr phosphorylation. The question of whether the EPO-R might complex with signalling molecules in the ER is thus of great interest. To address this point we examined whether Tyr-phosphorylated ER-retained  $\Delta$ WS1 EPO-R immunoprecipitates with JAK2. Cells expressing wt EPO-R or  $\Delta$ WS1 EPO-R were incubated with PV for 10 min, and cell lysates were immunoprecipitated with anti-JAK2 antibodies and resolved by



**Figure 7** Kinetics of EPO-R and JAK2 Tyr phosphorylation after treatment with PV

Ba/F3 cells expressing wt EPO-R were incubated at 37 °C with either EPO or PV for the indicated periods. Cell lysates were prepared and immunoprecipitated with either anti-(EPO-R) antibodies directed against the cytoplasmic region of the EPO-R (A) or anti-JAK2 antibodies (B). The immunoprecipitates were resolved by SDS/PAGE [7.5% (w/v) gel] followed by transfer to nitrocellulose membrane filters and incubation with anti-(P-Tyr) (anti PY) antibodies.

SDS/PAGE. Incubation of the blot with anti-(P-Tyr) antibodies revealed that Tyr-phosphorylated EPO-R co-immunoprecipitated with Tyr-phosphorylated JAK2 (Figure 8A, lanes 2 and 4; P-Tyr EPO-R and P-Tyr JAK2 are indicated with an arrowhead and an arrow respectively). Immunoprecipitation with anti-(EPO-R) antibodies revealed only P-Tyr EPO-R and not P-Tyr JAK2 (Figure 8A, lanes 6 and 8). Western blotting of Figure 8A with anti-JAK2 antibodies (Figure 8B, lanes 1–4) and anti-(EPO-R) antibodies (Figure 8B, lanes 5–8) was performed to verify the efficiency of immunoprecipitation. Note that both P-Tyr JAK2 (Figure 8B, lanes 2 and 4) and P-Tyr EPO-R (Figure 8B, lanes 6 and 8) migrated with a lower mobility,



**Figure 8**  $\Delta$ WS1 EPO-R co-immunoprecipitates with JAK2 on treatment with PV

Ba/F3 cells expressing wt EPO-R or  $\Delta$ WS1 EPO-R were incubated at 37 °C with PV for 10 min. Cell lysates were prepared and immunoprecipitated with anti-JAK2 antibodies (lanes 1–4) or with anti-(EPO-R) antibodies (lanes 5–8). The blot was probed with anti-(P-Tyr) antibodies (A), and after stripping it was reacted with anti-JAK2 antibodies (B, lanes 1–4), or anti-(EPO-R) antibodies (B, lanes 5–8). The arrow points at Tyr-phosphorylated JAK2, and the arrowhead at Tyr-phosphorylated EPO-R. The positions of molecular mass markers (in kDa) are indicated at the right.

probably owing to their phosphorylation. The results of this experiment support the possibility that the EPO-R might complex with JAK2 along the secretory pathway.

## DISCUSSION

This study demonstrates that the Tyr residues in the intracellular domain of the EPO-R are phosphorylated after intact cells are treated with PV. While previous studies have shown that only cell-surface EPO-R is Tyr phosphorylated after EPO binding [3,5,6,8], this study presents the novel observation that in addition to mature endo H-resistant EPO-R, intracellular EPO-R, localized in the ER, undergoes PV-mediated Tyr phosphorylation in a time-dependent and dose-dependent manner. Two experimental approaches support this conclusion. First, we have shown that after short periods of chase in PV-treated metabolically labelled cells, endo H-sensitive EPO-R is phosphorylated. Secondly, we demonstrated that an ER-retained EPO-R mutant,  $\Delta$ WS1 EPO-R, which cannot bind EPO [24], becomes Tyr phosphorylated as a result of treatment with PV while remaining endo H-sensitive, thereby indicating that it is probably phosphorylated in the ER, possibly by an ER protein Tyr kinase (PTK). PV-mediated phosphorylation of the EPO-R occurs largely in intracellular compartments, because phosphorylated EPO-R can be detected even after treatment of cells with proteinase K (100  $\mu$ g/ml for 20 min at 37 °C), which cleaves cell-surface EPO-Rs (results not shown). Indeed, the presence of signalling molecules in association with the ER is gaining support. Shc proteins were recently localized in the ER and were found to redistribute after Tyr kinase receptor activation [29]. The intracellular phosphatase PTP1B was shown to reside on the ER membrane facing the cytosol. Because this phosphatase is devoid of a transmembrane region, it is associated with the ER membrane via non-covalent interaction with ER membrane proteins [30]. In another study an ER kinase (Ltk) was identified, its activity being regulated by the redox potential within the ER [31]. In particular, it was shown that Ltk is activated by oxidation. PV, employed in this study, is a powerful oxidizing agent [14]; however, the lack of effect of H<sub>2</sub>O<sub>2</sub> on EPO-R phosphorylation argues that activation of PTKs by oxidation might be necessary but is not sufficient to result in Tyr phosphorylation of the EPO-R. Thus PV might activate PTKs as well as inhibiting PTPs, and this synergistic effect might be required to phosphorylate the EPO-R. The presence of both kinases and phosphatases in the ER lends support to our findings that the EPO-R might be phosphorylated in the ER. We have shown that after early chase periods of PV-treated metabolically labelled cells (Figure 5), the phosphorylated EPO-R is in its endo H-sensitive form. However, when the chase periods are prolonged, PV-mediated phosphorylation occurs mainly on endo H-resistant EPO-R. The fact that intracellular EPO-R is phosphorylated supports the idea that it is associated, while in the secretory pathway, with a PTK. EPO-R might thus consist of at least two populations, one that is complexed with a PTK and the other that is not, possibly owing to non-stoichiometric amounts of EPO-R and the putative PTK. Alternatively, inefficient folding of the EPO-R [27] might prevent it from generating high-affinity complexes with the intracellular PTK. A possible scenario is that EPO-R molecules that are targeted for exit from the ER are in a proper conformation to become substrates for PTKs. PV might thus serve as a unique probe to distinguish EPO-R molecules that are already complexed with kinase(s), and are on their way towards exiting from the ER, from other ER-retained EPO-R molecules that remain endo H-sensitive throughout the chase and do not become phosphorylated on stimulation with PV. The phos-

phorylation of the ER-retained EPO-R mutant  $\Delta$ WS1 might point out that this mutation does not affect its capacity to interact with Tyr kinase(s); however, this is not sufficient to mediate its exit from the ER.

The 39 kDa proteolytic fragment of the EPO-R, which contains the entire cytoplasmic domain, is not phosphorylated after stimulation with PV. This could be attributed to its inaccessibility to cellular kinases owing to its different cellular location. Although the full-length EPO-R is present at steady state at various intracellular locations (ER, Golgi, cell membrane and lysosomes), the fragment is formed in a cellular compartment distal to the *trans*-Golgi network and accumulates in the lysosomes [23]. The fragment might thus be generated at a cellular site that is inaccessible to PTKs. Alternatively, only whole non-cleaved EPO-R molecules might maintain the proper conformation required for phosphorylation. Another possibility is that the full-length EPO-R, associated with a PTK and subsequently phosphorylated, is not cleaved to generate the 39 kDa fragment. Although present at significant amounts at steady state, the 39 kDa fragment is usually detected only after 1 h of chase in metabolic labelling experiments, and accounts for the degradation of only a minor fraction of EPO-Rs [23]. Because PV is applied to the cells for 10 min, the kinetics of generation of the 39 kDa fragment might explain why the phosphorylated fragment cannot be detected. The existence of at least two populations of EPO-Rs is strongly implied by lack of phosphorylation of the 39 kDa fragment.

JAK2 is probably the first kinase that is activated after EPO binds to its cell surface receptor [7]. JAK2 activation requires the presence of Trp-282 in the intracellular domain of the EPO-R, close to the transmembrane region [28]. In this study we have shown that W282R EPO-R, which is not Tyr phosphorylated after EPO binding (Figure 6) [28], can become phosphorylated after exposure of the cells to PV. Under similar conditions JAK2 was also Tyr phosphorylated, with similar kinetics to the PV-mediated phosphorylation of EPO-R. These findings suggest that non-ligand-mediated activation of JAK2 can induce W282R EPO-R phosphorylation, yet the possibility that PV-mediated phosphorylation occurs via kinase(s) such as Src [32] cannot be ruled out. It was previously shown [3] that W282R EPO-R does not associate with JAK2 *in vivo*. However, the cytoplasmic domain of the W282R EPO-R, when expressed as a glutathione S-transferase fusion protein, could bind JAK2 kinase [7]. Thus the phosphorylation of JAK2 might increase its binding affinity for W282R EPO-R, leading to phosphorylation of this EPO-R.

Haemopoietic cytokines including EPO, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, IL-3 and IL-2 were shown to induce phosphorylation of intracellular proteins, after binding to their cell-surface receptors [33–40]. Evans et al. [18] have shown that PV, at lower concentrations than we have employed, can mimic some of the effects of IL-2. The authors concluded that PV probably does not activate JAK3, as judged by the inability of JAK3 to immunoprecipitate with anti-(P-Tyr) antibodies after treatment with PV [18]. Detection of JAK2 phosphorylation in the present study was obtained by immunoprecipitation with anti-JAK2 antibodies, followed by immunoblotting with anti-(P-Tyr) antibodies. The difference in the experimental systems and PV conditions, as well as the mode of detection of the phosphorylated JAK, might account for this apparent discrepancy. Our results are also supported by the observation that JAK2 is Tyr phosphorylated in thrombocytes after treatment with PV [41]. The finding that ER-retained  $\Delta$ WS1 EPO-R co-immunoprecipitates with JAK2 suggests that the EPO-R might complex with JAK2 in the ER. The question of whether JAK2 does indeed phos-

phorylate the EPO-R on treatment with PV is not yet resolved. Association of EPO-R with JAK2 on treatment with PV suggests that PV might mimic EPO-mediated activation. It is of importance to determine whether PV induces specific EPO-mediated events in Ba/F3 cells expressing the EPO-R. Experiments are also under way to explore whether treatment with PV leads to an association of intracellular, ER-retained EPO-R with other signalling proteins (e.g. STAT 5 and phosphoinositol 3-kinase).

It has not been determined whether similar Tyr residues on the EPO-R are phosphorylated after treatment with PV and after EPO binding. However, our studies show that stimulation with PV can be used as a powerful tool to analyse the intracellular association of cytokine receptors such as the EPO-R with PTKs along the secretory pathway. Hence these findings could have more general implications in the study of the assembly of signalling complexes of other cytokine receptors.

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