Tyrosine Phosphorylation of the Erythropoietin Receptor: Role for Differentiation and Mitogenic Signal Transduction

By Stéphanie Gobert, Françoise Porteau, Sylvie Pallu, Odile Muller, Michèle Sabbah, Isabelle Dusanter-Fourt, Geneviève Courtiois, Catherine Lacombe, Sylvie Gisselbrecht, and Patrick Mayeux

The erythropoietin (Epo) receptor belongs to the cytokine receptor superfamily. Although the cytokine receptors do not possess a tyrosine kinase consensus sequence in the intracellular domain, rapid stimulation of a tyrosine kinase activity occurs after activation by the ligand. We and others have shown that Epo induces the tyrosine phosphorylation of its cognate receptor as well as phosphorylation of other proteins. In this report, we examined the role of the receptor tyrosine residues in signal transduction. Eight tyrosine residues are located within the intracellular domain of the murine Epo receptor. A single tyrosine residue is present in the region previously shown to be sufficient for proliferative signal transduction. This tyrosine (Tyr 343) was mutated to phenylalanine. Moreover, mutant receptors were also generated with either a tyrosine residue or a phenylalanine residue at position 343 and with a COOH terminal truncation that removed the 7 other tyrosine residues. Expression vectors carrying these mutated receptors were transfected into the interleukin-3-dependent murine cell line Ba/F3. Epo-induced growth was sustained efficiently by all these receptors, although receptors without any tyrosine residues conferred a significantly reduced mitogenic activity. Moreover, all receptors were able to mediate Epo-dependent accumulation of β-globin mRNA. The mutated receptors all induced the tyrosine phosphorylation of several cellular proteins after Epo stimulation. However, the truncated receptors induced the phosphorylation of a reduced number of proteins, suggesting that phosphorylated tyrosines of the receptor could have a role in the recruitment either of a tyrosine kinase or of tyrosine kinase substrate proteins. The receptors were all able to mediate Epo-induced activation of phosphatidylinositol 3-kinase, although truncated receptors no longer bound phosphatidylinositol 3-kinase.

© 1995 by The American Society of Hematology.

Several tyrosine kinases have been implicated in the mechanism of cytokine receptor action and the Jak family of kinases appears to play a central role in the signalling of most, if not all, receptors of the cytokine receptor family. Activation of these kinases requires receptor subdomains also necessary for induction of cell proliferation as described for the Epo-R and overexpression of a kinase-deficient form of Jak2 abrogates Epo-induced mitogenesis. As with the receptors with intrinsic tyrosine kinase activity such as platelet-derived growth factor (PDGF), stem cell factor (SCF), or CSF-1 receptors, the cytokine receptors themselves are most generally tyrosine phosphorylated after ligand stimulation. With others have shown that the Epo-R is tyrosine phosphorylated after ligand binding. Although the Epo-R seems to be a multimeric complex, only the cloned chain is tyrosine phosphorylated. Receptor tyrosine phosphorylation plays a key role for signal transduction in the case of the receptors with intrinsic tyrosine kinase activity. Indeed, the phosphorylated tyrosines constitute binding sites for the SH2 domains of effector proteins such as src, phosphatidylinositol 3-kinase (PI 3-kinase), and phospholipase Cγ and for adaptor proteins allowing the stimulation of the ras pathway. These tyrosine residues are essential to transduce mitogenic signals. In the case of the cytokine receptors, the importance of the receptor tyrosines appears to be less clearly defined. Truncated granulocyte colony-stimulating factor (G-CSF) receptors lacking tyrosine residues in their intracellular domains remain able to transduce a mitogenic signal but do not induce the expression of specific genes. Most of the phosphorylated tyrosines of the Epo-R are located terminally in the intracellular domain, in a region previously described as unnecessary for proliferative signal transduction. However, one tyrosine residue (Tyr 343) is located closer to the transmembrane region. Deletion of the region of the Epo-R around Tyre 343 has been shown to decrease or nearly abrogate Epo-induced mitogenesis. The role of tyrosine phosphorylation in the transduction of a differentiation signal has never been tested. To investigate the role of the receptor tyrosine phosphorylation in Epo signal trans-
duction, we mutated or removed part or all the tyrosines of the intracellular domain of the Epo-R. Our results show that Epo-R tyrosine phosphorylation is dispensable for proliferative signal transduction, although receptors without any tyrosine residue in their intracellular domain were slightly less efficient. Moreover, all the mutated receptors were able to mediate Epo-stimulated accumulation of \(\beta\)-globin mRNA.

**MATERIALS AND METHODS**

*Reagents.* The monoclonal antiphosphotyrosine antibody 4G10 was a generous gift of Dr B. Drucker (Dana-Farber Cancer Institute, Boston, MA). Rabbit anti–Epo-R antibodies were produced as previously described and were affinity purified using an agarose-bound antigen column. They immunoprecipitated all the Epo-R mutants and were affinity purified using an agarose-bound antibody column. They immunoprecipitated all the Epo-R mutants and were affinity purified using an agarose-bound antibody column.

*DNA constructs and expression vectors.* The intracellular domain of the murine Epo-R contains 8 tyrosine residues (positions 343, 401, 429, 431, 443, 460, 464, and 479) of the mature protein; Fig 1). Tyrosine 343 was mutated to a phenylalanine residue by changing the TAC 343 codon into "ITC using the Kunkel method. The mutation Tyr343 was mutated to a phenylalanine residue by changing the TAC 343 codon into "ITC using the Kunkel method. For this mutation, a BamHI-HindIII restriction fragment was first cloned in pBluescript KS, mutated, and sequenced. An entire Epo-R was reconstituted with the mutagenized fragment and cloned between the Xba I and Cla I sites of pBluescript KS. To remove all the tyrosine residues of the COOH end of the receptor, HindIII-Cla I fragment was excised and an adaptor with a stop codon and an EcoRI site was inserted. Finally, all the receptors were cloned between the Kpn I and EcoRI sites of the pXM expression vector.

*Cell cultures.* Nontransfected Ba/F3 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 5% WEHI conditioned medium as a source of IL-3. After transfection and selection, Epo-sensitive cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 2 U/ml Epo. Serum-free cultures were performed in RPMI 1640 medium containing 0.4% bovine serum albumin (BSA; Sigma ref. A-8806; Sigma, St Louis, MO), 40 \(\mu\)g/ml iron saturated human transferrin and 5 U/ml Epo. For signal transduction experiments, the cells were grown using 1% WEHI conditioned medium and were starved for 2 to 5 hours by incubation in RPMI 1640 medium containing 0.4% BSA and 20 \(\mu\)g/ml iron-saturated transferrin. For Epo-R measurements, the cells were also cultured with WEHI conditioned medium but the starvation period was omitted.

*Stable transfections.* Ba/F3 cells were transfected by electroporation using a Bio-Rad (Heracles, CA) gene pulser set at 250 V and 960 \(\mu\)F. Fifty micrograms of pXM and 10 \(\mu\)g of a plasmid carrying a puromycin or a neomycin resistance gene were used in each transfection. After 48 hours of incubation into IL-3-containing medium, the cells were selected for puromycin or G418 resistance in the presence of IL-3. Simultaneously, cells were also selected for growing in the presence of 2 U/ml of Epo after IL-3 removal. In each transfection experiment, control cells transfected with the resistance gene alone or without plasmid were also submitted to selection. Except when otherwise indicated, all reported results were obtained using cells selected for their ability to grow under Epo stimulation.

*Cell proliferation assays.* Cell proliferation was measured by the colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using 5 \times 10^5 cells per well in 96-well plates and a 4-hour pulse of MTT after 72 hours of culture with the growth factors.

*Receptor measurement.* Epo was iodinated using Iodogen (Pierce, Rockford, IL), as previously described, with specific radioactivities ranging from 30 to 60 \(10^6\) cpm/\(\mu\)g. Epo binding experiments, Scatchard analysis, and chemical cross-linking were performed as previously described.

*Immunoprecipitation and Western blotting.* Immunoprecipitations and Western blots were performed as previously described. ECL (Amersham Ltd, Les Ullis, France) was used for the Western blot revelations.

*PI 3-kinase assays.* PI 3-kinase assays were performed using antiphosphotyrosine immunoprecipitates as previously described. Association between PI 3-kinase and Epo-R was tested by immunoprecipitation of receptor-bound I^125-I-Epo as previously reported.

*Globin mRNA measurements.* RNA extraction and Northern blot analysis were performed as previously reported.

**RESULTS**

*Mitogenic activity of Epo-R mutants.* To explore the role of the tyrosine residues of the cytoplasmic domain of the Epo-R, we constructed three Epo-R mutants (Fig 1). Tyr343 in the cytoplasmic domain of the wild-type Epo-R was substituted by a phenylalanine residue to create the "Phe" mutant. The "Stop" mutant retained only Tyr343 after removal of the 109 last amino acids. The "Zero" mutant did not possess any tyrosine residue in the intracellular domain after the mutation to phenylalanine of the Tyr343 in the Stop mutant. These three mutants as well as the wild-type Epo-R (N) were cloned in the expression vector pXM and introduced by electroporation into Ba/F3 cells. The pXM plasmids carrying the mutated receptors were cotransfected with pBabe plasmids carrying either a neomycin or a puromycin resistance gene. Transfected cells were first selected for G418 or puromycin resistance in the presence of IL-3 and then tested for their ability to grow in the presence of Epo after IL-3 removal. Cells able to grow in Epo were obtained from Ba/F3 cells transfected with normal receptors or with the Phe or the Stop mutants but not from cells transfected with the
obtained in 5 independent transfections. Epo-sensitive cells
mutant were immediately selected for Epo sensitivity instead
regardless of the selection method and untransfected cells
transfected with the neomycin or puromycin selection vector
could never be isolated from untransfected cells or from cells
cells transfected with the Zero mutant was slightly depressed.
with the wild-type receptor, whereas the Epo sensitivity of
mutants conferred Epo sensitivity similar to that observed
with the wild type Epo-R;
2). Epo dose-response curves showed that the Phe and Stop
mutant; (+) Ba/F3 cells transfected with the Stop mutant; (○) Ba/
F3 cells transfected with the Zero mutant. All transfected cells were
previously selected for growth under Epo stimulation.

Zero mutant. However, when cells transfected with the Zero
mutant were immediately selected for Epo sensitivity instead
of antibiotic resistance, cells able to grow in Epo were always
obtained in 5 independent transfections. Epo-sensitive cells
could never be isolated from untransfected cells or from cells
transfected with the neomycin or puromycin selection vector
regardless of the selection method and untransfected cells were
Epo unresponsive even at high Epo concentrations (Fig
2). Epo dose-response curves showed that the Phe and Stop
mutants conferred Epo sensitivity similar to that observed
with the wild-type receptor, whereas the Epo sensitivity of
cells transfected with the Zero mutant was slightly depressed.
To determine whether our results were also applicable to
other IL-3–dependent cell lines, FDCP-1 cells were
transfected with the wild-type Epo-R or the Zero mutant and
transfected cells were selected for their ability to grow in
Epo. In both cases, Epo-sensitive cells were obtained. More-
over, as observed for Ba/F3 cells, FDCP-1 cells transfected
with the Zero Epo-R mutant showed an Epo sensitivity lower
than that found in cells transfected with the wild-type Epo-
R (data not shown). In serum-free medium, Epo was also
able to sustain long-term growth (>3 weeks) of Ba/F3 cells
transfected with each of the receptor forms (data not shown).
The Zero mutant differed from the Stop mutant only by a
single base mutation; to rule out the possibility that re-
vertants had been selected, mRNAs from Epo-sensitive cells
were isolated and the sequence encoding the intracellular
domain was reverse-transcribed, amplified by polymerase
chain reaction (PCR), and sequenced. Cells transfected with
the Phe mutant were likewise analyzed. The sequences did
not show any modification from the transfected cDNA, demo-
strating that no reversion had occurred.

Epo-induced β-globin mRNA accumulation. It has been
previously described that Ba/F3 cells transfected with Epo-
R synthesized β-globin mRNA when the cells were grown
in Epo.31-32 We tested the accumulation of β-globin mRNA
in Ba/F3 cells transfected with normal or mutated Epo-Rs
and grown for 3 weeks either under Epo or under IL-3 stimu-
lation. As shown on Fig 3, all mutated receptors were able to
induce β-globin gene expression when the cells were
stimulated by Epo but not when the cells were stimulated by
IL-3. The same results were also obtained in two independent
experiments showing that Epo-induced differentiation of Ba/
F3 cells is not dependent on tyrosine phosphorylation of the
Epo-R.

Characterization of the cell surface receptors. Scatchard
analyses were performed using cells selected for ability to
grow under Epo stimulation. The analysis showed that the
cells transfected with the wild-type receptor, the Phe mutant,
or the Stop mutant expressed similar numbers of Epo-Rs. In
contrast, Epo-sensitive cells transfected with the Zero mutant
expressed a significantly higher number of cell surface Epo-
Rs (Table 1). Cells transfected with the Zero mutant and
selected for neomycin resistance did not exhibit an in-
creased number of Epo-Rs (data not shown), suggesting that
the increased number of receptors is the result of the Epo
selection. The affinity of the Epo binding sites was slightly
lower for the cells transfected with the Zero mutant (kd =
1.6 ± 0.4 nmol/L) than for the cells transfected with the
other receptor forms (kd = 0.6 to 0.7 nmol/L). Non-
transfected cells or cells transfected with the neomycin resis-
tance gene alone did not exhibit detectable Epo-specific
binding. Cross-linking experiments were performed using
dissuccinimidyl suberate (DSS) to cross-link Epo with its
receptor (Fig 4). Two Epo cross-linked proteins can be seen
in cells transfected with each receptor form. The molecular
mass of the lower band was decreased in the cells transfected
with the truncated receptors (p46 in Stop or Zero compared
with p66 in N and Phe) and corresponded to the transfected
Epo-R chain as previously reported.6 The molecular mass of
the upper band (120 kD) did not change after transfection
with the truncated receptor, showing that this protein did not
derive from the transfected cDNAs and is compatible with
the cross-linking of one Epo molecule (34 kD) with a 85-
kD protein similar to the Epo-R-associated protein already
described in most erythroid cells.2-6

Tyrosine phosphorylation stimulated by Epo. As shown
on Fig 5, Epo was able to induce the tyrosine phosphoryla-
tion of several proteins in Ba/F3 cells transfected with each
receptor form but not in nontransfected cells. In cells
transfected with full-length Epo-Rs (N and Phe), Epo stimu-
lated the tyrosine phosphorylation of proteins of 145, 130,
115, 98-90, 85, 72, 60, 52, and 48 kD molecular masses. In
cells transfected with truncated receptors (Stop or Zero), the overall tyrosine phosphorylation pattern induced by Epo was strongly reduced and Western blots mainly showed the Epo-stimulated tyrosine phosphorylation of the 145- and the 130-kD proteins. Moreover, the tyrosine phosphorylation of protein(s) migrating as a diffuse band between 98 and 90 kD was also stimulated by Epo in cells transfected with the Stop mutant, whereas it was barely detectable in cells expressing the Zero mutant. To investigate whether the Jak2 kinase corresponded to the 130-kD tyrosine phosphorylated protein, cell lysates immunoprecipitated with an anti-Jak2 antibody were analyzed by Western blot using antiphosphotyrosine antibodies. As shown in Fig 6, Jak2 was tyrosine phosphorylated in Epo-stimulated BalF3 cells transfected with the normal or the mutated Epo-Rs.

To study the tyrosine phosphorylation of the Epo-R mutants characterized by a reduced number of tyrosine residues, several experiments were performed. First, Epo-Rs from Epo-stimulated or unstimulated cells were immunoprecipitated using anti-Epo-R antibodies and the immunoprecipitated material was analyzed by Western blot using antiphosphotyrosine antibodies. Only the tyrosine phosphorylation of N and Phe receptors could be seen (Fig 7A). Biotinylated Epo was also used to stimulate BalF3 cells transfected with the wild-type (N) receptor or the Stop mutant and the Epo–Epo-R complexes were precipitated using agarose-bound streptavidin. Western blots analysis of precipitated material using antiphosphotyrosine antibodies showed the tyrosine phosphorylation of the normal Epo-R but not the tyrosine phosphorylation of the Stop mutant (Fig 7B). Finally, we immunoprecipitated 125I-Epo cross-linked to its receptor using antiphosphotyrosine antibodies after denaturation of the receptor complex. Again, only N and Phe but not Stop and Zero receptors were recognized by antiphosphotyrosine antibodies (data not shown). Thus, by these three methods, Tyr of the Stop mutant appeared to be nonphosphorylated.

**PI 3-kinase activation by Epo.** Activation of PI 3-kinase has been reported to be involved in signal transduction pathway induced by Epo. As shown in Fig 8, all mutated receptors, including the Zero mutant, were able to mediate Epo stimulation of the PI 3-kinase activity. The association between PI 3-kinase and normal or mutated Epo-Rs was tested by immunoprecipitation of 125I-Epo bound to its receptor using anti–PI 3-kinase antibodies. Anti–PI 3-kinase antibodies immunoprecipitated Epo-R–bound 125I-Epo from cells transfected with the normal Epo-R or the Phe mutant but not from cells transfected with the Stop or the Zero mutants (Fig 9). Thus, although all mutated receptors mediate Epo-induced activation of the PI 3-kinase, PI 3-kinase associates only with the full-length Epo-Rs (N and Phe) but not with the Stop or the Zero mutants.

**DISCUSSION**

Tyrosine phosphorylation of receptors with intrinsic tyrosine kinase activity plays a key role in mitogenic signal transduction. Indeed, mutation of the tyrosines of the PDGF or the M-CSF receptor fully abolishes their mitogenic potential. Most cytokine receptors are also tyrosine phosphorylated after ligand binding, but the role of this phosphorylation is less clearly understood. Our results show that tyrosine phosphorylation of the Epo-R is not required for long-term cell proliferation of BalF3 cells, even in serum-free conditions. However, Epo-responsive BalF3 cells transfected with a receptor devoid of tyrosine residues in the cytoplasmic domain exhibited a lower Epo sensitivity and expressed an higher number of Epo-Rs than did BalF3 cells transfected with the other tested Epo-Rs. The data presented in Fig 2...
of Tyr^345. In contrast, the Stop mutant that only retained the Tyr^345 conferred to transfected cells an Epo sensitivity similar to that conferred by the wild-type receptor. However, the only difference between Stop and Zero mutants is Tyr^345, which did not appear to be phosphorylated in Ba/F3 cells. We cannot rule out that the tyrosine to phenylalanine replacement could modify the local conformation of a region important for signal transduction. However, this latter explanation seems unlikely because the Phe mutant that carried the same substitution conferred to transfected cells an Epo sensitivity similar to that conferred by the wild-type receptor. Alternatively, Tyr^345 could be tyrosine phosphorylated in the Stop mutant, but our methods were not sensitive enough to allow its detection. Interestingly, the sequence after the Tyr^345 (YLVL) of the Stop mutant is close to that after the tyrosine residues 429 (YLYL) or 431 (YLVV) of the Phe mutant; it is therefore possible that compensatory mechanisms could occur when one or the other tyrosine is removed in the Phe or Stop mutant. Such a mechanism was recently described for the epidermal growth factor receptor. A recent paper of Yoshimura reported the expression of endogenous Epo-R in Ba/F3 cells expressing chimaeric receptors with Epo-binding ability. As shown in Figs 4, 5, 7, and 9, our results did not evidence the expression of endogenous Epo-Rs in cells transfected with the Stop and Zero truncated receptors. Taking into account the sensitivity of the methods used in these experiments, we can assume that Ba/F3 cells

and Table 1 indicated that half-maximum stimulation of cell growth required the occupancy of 10-fold more Zero mutant receptors than wild-type receptors. Previous studies using deletion mutants have shown that Epo-Rs truncated before Tyr^336 were unable to transduce a mitogenic signal in DA-3 cells, but they stimulated the proliferation of LyD9 cells, another pro-B-cell line, with a reduced efficiency. These discrepancies could reflect different requirements for these two cell lines to proliferate but, according to our results, they could also reflect different selection mechanisms of Epo-sensitive cells. The reduced efficiency of the truncated Epo-R mutant in LyD9 cells is similar to that we observed for the Zero mutant and could be explained by the absence of Tyr^345.
transfected with the Stop or the Zero mutant Epo-R and selected for their ability to grow in Epo would express less than 30 to 50 endogenous receptors per cell, if any.

All mutant receptors were able to associate with the 85-kD Epo-R accessory protein (p85) previously described in erythroid cells. We have shown that antibodies directed against the intracellular domain of the cloned chain of the Epo-R do not directly recognize p85. This finding suggests that p85 is associated with but is not related to the cloned chain. The apparent molecular mass of p85 did not change after cell transfection with a truncated Epo-R (Fig 4), confirming that this protein did not derive from the transfected cDNA. Epo cross-linking to this protein was not increased in cells overexpressing Epo-R like the cells transfected with the Zero mutant, suggesting that this protein could be expressed in limiting amounts in Ba/F3 cells.

It has been shown that Ba/F3 cells first described as pro-B cells39 seems to be rather close to erythroid cells.40 Indeed, these cells also expressed erythroid-specific transcription factors such as GATA-1 and SCL and synthesized β-globin mRNA when Epo-R–transfected Ba/F3 cells were grown in Epo.31,32 Our results confirm these observations and we show that all receptor mutants, including the Zero mutant, were able to mediate Epo-stimulated β-globin accumulation. Thus, neither the receptor tyrosine phosphorylation nor the receptor COOH terminal region appeared to be required for Epo-induced erythroid differentiation. Using chimeric receptors, Chiba et al33 have shown that the extracellular domain of the Epo-R was essential for erythroid differentiation. These investigators concluded that the interaction of the extracellular domain of the Epo-R with other membrane components was essential for the transmission of erythroid differentiation signal; the 85-kD Epo cross-linked protein associated with all our Epo-R mutants could be such a protein.

In Ba/F3 cells transfected with the full-length receptors (wild-type or Phe mutant), Epo stimulated the tyrosine phosphorylation of proteins of 145, 130, 115, 98–90, 85, 72,
Fig 8. PI 3-kinase activation by normal and mutated Epo-Rs. Ba/F3 cells transfected with normal or mutated Epo-Rs were stimulated for 5 minutes with 5 U/mL Epo. The cells were then solubilized and tyrosine phosphorylated proteins were immunoprecipitated using antiphosphotyrosine monoclonal antibodies. Immunoprecipitated material was incubated with \(^{32}\)P-yATP and phosphatidylinositol as previously described. Phospholipids were then extracted and separated by thin-layer chromatography. ORI and PIP show the origin and migration position of unlabelled PI 4-phosphate run in an adjacent lane and shown by iodine vapor staining. In this chromatographic system, PI 4-phosphate and PI 3-phosphate are not separated.

Fig 9. Association between PI 3-kinase and wild-type or mutated Epo-Rs. Ba/F3 cells transfected with normal or mutated Epo-Rs were stimulated for 10 minutes with 1 nmoL \(^{125}\)I-Epo (nearly 4 U/mL), washed to remove unbound radioactivity, and lysed using 1% Nonidet P40, and the extracts were centrifuged for 20 minutes at 25,000 g. Aliquots of the supernatants were immunoprecipitated using either anti-Epo-R or anti-PI 3-kinase antibodies. Anti-Epo-R antibodies immunoprecipitated \(^{125}\)I-Epo–Epo-R complexes with the same efficiency regardless of the Epo-R mutant (data not shown). The results are expressed as percentage of radioactivity immunoprecipitated by anti-Epo-R antibodies and are means ± standard deviations from three independent experiments.

60, 52, and 48 kD. This pattern of Epo-induced tyrosine phosphorylation is close to that previously reported for the human cell line UT7 and for B6SUt.EP cells. The 72-kD protein is the Epo-R itself (Fig 7) and at least part of the 130-kD band corresponded to the Jak2 kinase (Fig 8). However, in agreement with other studies, much less protein appeared to be tyrosine phosphorylated in cells transfected with the truncated mutants (Stop or Zero), suggesting that a tyrosine kinase and/or protein kinase substrates associate with the distal part of the receptor. Because the truncated receptors transduce both proliferative and differentiation signals, it appears that most of these tyrosine phosphorylated proteins are necessary neither for cell proliferation nor for erythroid differentiation in Ba/F3 cells. In cells transfected with the truncated receptor mutants, Epo mainly induced the tyrosine phosphorylation of the Jak2 kinase and of proteins of 145 and 90-98 kD molecular masses, although the tyrosine phosphorylation of the 90-98–kD proteins was strongly decreased in cells transfected with the Zero mutant. The 145-kD protein could correspond to that associated with the Shc protein in Epo-stimulated DA-ER cells and the 90-97-kD protein(s) could correspond to the unidentified cytosolic factor (pp100) previously reported to be tyrosine phosphorylated after Epo stimulation. However, unlike Jak2, specific probes for pp100 and the 145-kD phosphoprotein have not yet been developed. The Jak2 kinase was previously shown to associate with a region of the Epo-R close to the transmembrane domain, which is conserved in all our mutants. However, although Jak2 activation appears to be necessary for Epo signal transduction, it has been shown to be not sufficient for efficient mitogenesis, which also requires a region of the receptor including Tyr. Additional transduction pathway(s) activated by Epo via these truncated receptors should be necessary for efficient mitogenesis.

To tentatively identify these transduction pathways, we studied PI 3-kinase activation in Ba/F3 cells transfected with the Epo-R mutants. PI 3-kinase activation is required for cell
proliferation activated by several growth factors. We and others have reported Epo-induced stimulation of PI 3-kinase and its association with the tyrosine phosphorylated Epo-R. However, the mechanism of Epo-induced PI 3-kinase stimulation remains controversial. According to He et al., PI 3-kinase binds to the activated Epo-R through the phosphorylated tyrosine residues of the carboxyl-terminal region, thus to a region dispensable for Epo-stimulated mitogenicity. Our results show that PI 3-kinase associates with the carboxyl-terminal region of the Epo-R and that tyrosine phosphorylation is not involved in this binding. But our results also show an alternative way for PI 3-kinase activation because Epo also activates this enzyme in Ba/F3 cells expressing the Zero or the Stop Epo-R mutants that do not associate with PI 3-kinase. In view of our results, it is now necessary to determine the importance of PI 3-kinase activation in Epo mechanism of action.

ACKNOWLEDGMENT

We thank Fatima Ferrag for her help in the study of Jak2 phosphorylation and Drs. Simon and Véronique Duprez for helpful discussion. The excellent technical work of Karine Goude is greatly acknowledged.

REFERENCES

20. Van der Goot P, Hunter T: Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. EMBO J 12:5161, 1993
31. Liboi E, Carroll M, D’Andrea AD, Mathey-Prevot B: Erythropoietin

From www.bloodjournal.org by guest on October 31, 2017. For personal use only.
erythropoietin receptor signals both proliferation and erythroid-specific differentiation. Proc Natl Acad Sci USA 90:11351, 1993


44. Roche S, Koegl M, Courtneidge S: The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors. Proc Natl Acad Sci USA 91:9185, 1994
Tyrosine phosphorylation of the erythropoietin receptor: role for differentiation and mitogenic signal transduction

S Gobert, F Porteu, S Pallu, O Muller, M Sabbah, I Dusanter-Fourt, G Courtois, C Lacombe, S Gisselbrecht and P Mayeux