Erythropoietin Induces Association of the JAK2 Protein Tyrosine Kinase With the Erythropoietin Receptor In Vivo

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Protein tyrosine phosphorylation has been hypothesized to play a key role in the growth signaling induced by erythropoietin (Epo), although the Epo receptor (EpoR), a member of the cytokine receptor superfamily, lacks a tyrosine kinase domain. Recently, the JAK2 tyrosine kinase was shown to be activated on Epo stimulation and to bind to the cytoplasmic domain of EpoR in vitro. To further explore the mechanisms of activation of JAK2 in EpoR-mediated signal transduction, we assessed the conditions for association of JAK2 with EpoR in vivo. Epo stimulation rapidly induced association of JAK2 with the EpoR in an interleukin 3 (IL-3)-dependent cell line transfected with the wild-type EpoR. On Epo stimulation JAK2 also associated with a truncated mutant EpoR (H-mutant), which is mitogenically active but not tyrosine phosphorylated, indicating that association does not require receptor phosphorylation and occurs in the membrane proximal region. However, association was not detected with mutant receptors inactivated by an internal deletion or a point mutation, Trp326 to Arg, in a membrane-proximal cytoplasmic region (PB or PM4 mutant, respectively). Immune complex kinase assays of anti-EpoR immunoprecipitates also revealed that activated JAK2 associates with the EpoR in Epo-stimulated cells. By this approach, association also occurred with the mitogenically active H mutant but not with the mitogenically inactive PB or PM4 mutants. In the immune complex kinases assays, EpoR, JAK2, and a 150-kD protein were phosphorylated on tyrosine. Taken together, the results further support the hypothesis that, on Epo stimulation, JAK2 associates with the membrane-proximal cytoplasmic region of the EpoR to be activated and induces tyrosine phosphorylation of cellular substrates, including the EpoR, to transduce a growth signal.

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ERYTHROPOIETIN (Epo) is a growth factor that regulates proliferation and differentiation of erythroid precursor cells.1,2 The receptor for Epo (EpoR),3-5 belongs to the cytokine receptor superfamily,6,7 which includes most of the receptors for hematopoietic growth factors. The members of this family are characterized by the presence of four positionally conserved cysteines and a Trp-Ser-X-Trp-Ser (WSXWS) motif in the extracellular domain. The intracellular domain of these receptors lacks any identifiable motifs that might indicate the signal transduction mechanisms, including a tyrosine kinase domain. However, many of the hematopoietic growth factors rapidly induce tyrosine phosphorylation of cellular protein substrates.8 In addition, dependency of hematopoietic cell lines on these growth factors is abrogated by a variety of activated tyrosine kinases.9 Thus, it has been hypothesized that tyrosine phosphorylation plays a critical role in the growth signal transduction by the hematopoietic growth factor receptors.

Using interleukin 3 (IL-3)-dependent cell lines transfected with the EpoR cDNA,10 we have previously shown that Epo rapidly induces tyrosine phosphorylation of a series of cellular substrates including EpoR. All of the proteins, except for EpoR, were also phosphorylated by IL-3 stimulation, suggesting that a common tyrosine kinase may be involved in the signal transduction from the receptors for these cytokines. We also showed that, among mutant EpoRs with carboxyl and internal deletions in the cytoplasmic region, the ability to induce tyrosine phosphorylation correlated with the ability to transduce the growth signal. On Epo stimulation, the EpoR undergoes tyrosine phosphorylation in the carboxyl-terminal region and associates with phosphatidylinositol 3-kinase.10-12 However, the carboxyl-terminal region of the receptor is not required for the growth signal transduction, because several carboxyl-truncated mutant EpoRs, including the H mutant with 108 amino acid deletion,13 retain the ability to elicit a mitogenic response.14,15 Conversely, a membrane-proximal cytoplasmic region, which shows homology with other cytokine receptors, was found to be essential for receptor function, because the abilities to induce tyrosine phosphorylation and mitogenesis were completely abolished in mutant receptors with an internal deletion of 20 amino acids or with a substitution of a single conserved amino acid, Trp326 to Arg, in this region (PB or PM4 mutant, respectively).15,16,17

JAK219,20 was first identified by polymerase chain reaction (PCR) amplification of tyrosine kinase domains in hematopoietic cells21,22 and belongs to the Janus family of cytoplasmic tyrosine kinases, which also includes JAK123 and Tyk2.24 The members of this family are characterized by the presence of a second kinase-like domain and the absence of SH2 and SH3 domains. Tyk2 has been implicated in the signal transduction mediated through the interferon alpha receptor.25 More recently, signaling through the interferon alpha receptor has been shown to also require JAK1,26 whereas signaling through the interferon gamma receptor requires both JAK1 and JAK2.27,28 The JAK family of kinases has also been shown to play a role in the signal transduction pathway from receptors of the cytokine receptor superfamily. In particular,
stimulation of responsive cells with Epo or IL-3 induces tyrosine phosphorylation of JAK2 and activates its in vitro kinase activity.\textsuperscript{20,27} JAK2 has also been implicated in signaling through the receptors for growth hormone\textsuperscript{46} and prolactin\textsuperscript{47}, whereas both JAK1 and JAK2 are involved in signaling through receptors that use the gp130 signaling receptor subunit.\textsuperscript{20,21,23} In the case of EpoR, there was correlation between the mitogenic activity of a series of mutants and the ability to phosphorylate and activate JAK2. In addition, JAK2 was shown to associate in vitro with the membrane-proximal cytoplasmic region of the EpoR, which is crucial for the signal transduction from the EpoR. Thus, it was hypothesized that JAK2 is the tyrosine kinase that mediates Epo binding to tyrosine phosphorylation and mitogenesis.

To extend these observations and better understand the mechanisms by which ligand binding activates JAK2, we examined the association of JAK2 with the EpoR in vivo. The present studies show that Epo rapidly induces the association of JAK2 with the mitogenically active EpoRs but not with the PB or PM4 mutant inactivated by an internal deletion or a point mutation in the membrane proximal region, respectively. Furthermore, immune complex kinase assays of anti-EpoR immunoprecipitates suggest that activated JAK2 represents the major kinase activity found associated with the activated EpoR.

MATERIALS AND METHODS

Cells and reagents. A clone of 32D cells, an IL-3-dependent cell line originally isolated from long-term bone marrow cultures\textsuperscript{7} has been previously described.\textsuperscript{23} 32D clones expressing the wild-type or various mutant EpoRs were also previously described.\textsuperscript{12,18} and maintained in RPMI 1640 medium supplemented with 10% calf serum and 10% WEHI-3 conditioned medium as a source of IL-3.

The preparation and properties of rabbit polyclonal antiserum against the cytoplasmic portion of recombinant murine EpoR and a peptide from JAK2 have been described.\textsuperscript{20,25} Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co, Ltd (Tokyo, Japan). Anti-phosphotyrosine monoclonal antibody (4G10) was purchased from UBI (Lake Placid, NY). Antibodies against Tyk2 were obtained from a commercial source (Santa Cruz Biotechnology Inc, CA).

Immunoprecipitation and immunoblotting. Cells were washed free of IL-3, cultured overnight, and left unstimulated as a negative control or stimulated with Epo or IL-3 at saturating concentrations.

Results

JAK2 becomes tyrosine phosphorylated and associates with the EpoR in cells stimulated with Epo. Previous studies\textsuperscript{27} have shown that Epo induces tyrosine phosphorylation and activation of JAK2 in Epo responsive cells. In addition, JAK2 was shown to bind in vitro with a recombinant fusion protein containing the intracellular domain of the EpoR. However these studies did not address the ability of EpoR to associate with JAK2 in vivo and the potential role of ligand binding in the association.

Consistent with our previous results,\textsuperscript{11,13,18} anti-phosphotyrosine blotting showed that Epo induced tyrosine phosphorylation of 150-, 130-, 92-, 72-, 70-, and 62-kD proteins in an IL-3-dependent cell line, 32D, expressing the transfected murine EpoR cDNA (Fig 1, TLC). IL-3 also induced a very similar pattern of tyrosine phosphorylation. However, IL-3 uniquely induced tyrosine phosphorylation of the β-subunit of its own receptor as a 140-kD protein, while a 72-kD form of the tyrosine-phosphorylated EpoR was observed only after Epo stimulation (data not shown). JAK2, immunoprecipitated from cells lysed with 1% digitonin, was tyrosine phosphorylated after Epo or IL-3 stimulation, consistent with our previous results\textsuperscript{27} and comigrated with the 130-kD phosphotyrosyl protein observed in the total cell lysates (Fig 1, aJAK2). In addition, a tyrosine-phosphorylated 72-kD protein was coimmunoprecipitated with JAK2 after Epo stimulation, thus suggesting that JAK2 associates with the tyrosine-phosphorylated EpoR in vivo. Antiphosphotyrosine blotting of anti-EpoR immunoprecipitates also showed a tyrosine-phosphorylated 130-kD protein that comigrates with JAK2, that associates with EpoR after Epo stimulation. By comparing the results of immunoprecipitation with
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Fig 1. JAK2 becomes tyrosine phosphorylated and associated with the EpoR in Epo-stimulated cells. 32D/EpoR-Wt cells were washed out of IL-3 overnight and left unstimulated (−) or stimulated with Epo (E) or IL-3 (IL) for 5 minutes before solubilization. Aliquots of cell lysates (TCL) (5 × 10^6 cells) and immunoprecipitates obtained from cell lysates (3 × 10^6 cells) with anti-JAK2 (αJAK2), anti-EpoR (αEpoR), or normal rabbit serum (NRS) were resolved by 6% SDS-PAGE and subjected to immunoblotting with an anti-phosphotyrosine monoclonal antibody, 4G10. The molecular weight markers are indicated and given in kilodaltons. The positions to which JAK2 and the tyrosine-phosphorylated EpoR (EpoR-PY) migrated are indicated with arrows. Major substrates of tyrosine phosphorylation after Epo stimulation are also indicated with filled circles.

As shown in Fig 3A, phosphorylated proteins of 72 and 130 kD were predominantly observed when the anti-EpoR immunoprecipitate from Epo-stimulated cells were subjected to in vitro kinase reactions. The 72- and 130-kD proteins were identified as EpoR and JAK2, respectively, by the secondary immunoprecipitation with specific antibodies (Fig 3B). Phosphoamino acid analysis showed that both were predominantly phosphorylated on tyrosine (Fig 3C). The immune complex kinase assays of anti-JAK2 immunoprecipitates also showed that Epo stimulation induced the activation of JAK2 and its in vitro phosphorylation of the associated EpoR. Together, these results strongly suggest that activated JAK2 represents the major tyrosine kinase activity that associates with and phosphorylates the EpoR.

Association of JAK2 with the EpoR in vivo correlates with the mitogenic ability of the receptor. To explore the functional significance of the association of JAK2 with EpoR in vivo, we next examined cell lines expressing various mu-

anti-JAK2 and those with anti-EpoR, it was evident that a substantial portion of the tyrosine-phosphorylated EpoR is associated with JAK2 after Epo stimulation, while only a small portion of tyrosine-phosphorylated JAK2 is associated with the EpoR. Though similar results were obtained when cells were lysed by using 1% Triton X-100 as a detergent, the amounts of co-immunoprecipitated proteins were consistently less than when 1% digitonin was used; therefore, digitonin was exclusively used in the subsequent studies.

To confirm the association of JAK2 with the EpoR and to examine whether JAK2 associates with the EpoR constitutively or inducibly after Epo stimulation, 32D/EpoR-Wt cells were stimulated for varying periods before solubilization, and anti-EpoR immunoprecipitates were analyzed by immunoblotting with anti-JAK2 (Fig 2). The association of JAK2 with the EpoR was not detected before Epo stimulation. However, JAK2 detectably coimmunoprecipitated with the EpoR within 1 minute after Epo stimulation, peaked from 2 to 30 minutes, and slowly declined thereafter. Reprobing of the membrane with anti-EpoR confirmed that equivalent amounts of EpoR were immunoprecipitated and showed that, upon Epo stimulation, a very small fraction of the EpoR was converted to the 72-kD form, which reacted intensely with antiphosphotyrosine antibody (Fig 2). Intriguingly, antiphosphotyrosine blotting also showed that, in addition to JAK2, a 92-kD tyrosine-phosphorylated protein coimmunoprecipitated with EpoR from 10 to 30 minutes after Epo stimulation.

Association of the JAK2 kinase activity with the EpoR and tyrosine phosphorylation of the EpoR in vitro. To further confirm that activated JAK2 associates with EpoR in vivo and to determine whether JAK2 phosphorylates EpoR in vitro, we next performed immune complex kinase assays of immunoprecipitates obtained with anti-EpoR or anti-JAK2.
tant EpoRs. 32D/EpoR-H cells express the truncated H mutant, which lacks the carboxyl-terminal 108 amino acids but retains the ability to transduce a mitogenic signal. 32D/EpoR-PB cells express the PB mutant, which is inactivated by an internal deletion of 20 amino acids in a membrane proximal region of the cytoplasmic domain. The 32D/EpoR-PM4 mutant contains an inactivating point mutation, Trp232 to Arg, in the membrane proximal region.

The cell lines expressing mutant EpoRs were first examined by anti-phosphotyrosine blotting of JAK2 immunoprecipitates with or without Epo stimulation. Consistent with our previous observation, the tyrosine phosphorylation of JAK2 was induced by Epo stimulation in cells expressing the H mutant, which retains the mitogenic function, but not in cells expressing the inactivated PB or PM4 mutant (Fig 4A). Moreover, the 72-kD EpoR was readily detectable in blots of extracts from cells expressing the wild-type receptor. Phosphorylation of EpoR was not detected in cells expressing the H mutant because this carboxyl-truncation removes the sites of tyrosine phosphorylation. Because the PB and PM4 mutants are not tyrosine phosphorylated following Epo stimulation, their ability to associate with JAK2 could not be addressed. Therefore, we examined anti-EpoR immunoprecipitates by blotting with antiphosphotyrosine and anti-JAK2. As shown in Fig 4B, the tyrosine-phosphorylated 130-kD protein was coimmunoprecipitated with the H mutant after Epo stimulation. Anti-JAK2 blotting further showed that the association of JAK2 with the H-mutant was dependent on Epo stimulation (Fig 4C). Importantly, however, JAK2 did not detectably co-immunoprecipitate with the mitogenically inactive mutants, including the PM4 mutant containing a single amino acid substitution in the critical membrane proximal region.

We next examined the Epo-induced association of JAK2 with the mutant receptors by the immune complex kinase assays of the anti-EpoR immunoprecipitates. As illustrated in Fig 5, in vitro kinase assays of EpoR immunoprecipitates of cells expressing the H mutant, resulted in the phosphorylation of a 130-kD protein that comigrates with JAK2, comparable with that seen in immunoprecipitates from cells expressing the wild-type receptor. However, the H mutant did not undergo tyrosine phosphorylation in vitro, consistent with the absence of putative tyrosine phosphorylation sites in the truncated mutant. It should also be noted that the ability of the H mutant to bind JAK2, shows that this association does not require tyrosine phosphorylation of the receptor. Neither JAK2 nor any other kinase activity was

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**Fig 3. Association of in vitro kinase activity with EpoR.** (A) Immune complex kinase assays of anti-EpoR and anti-JAK2 immunoprecipitates. After culture overnight without IL-3, 32D/EpoR-Wt cells were stimulated with Epo for 5 minutes (+) or left unstimulated (−). Cells were then lysed and equivalent amounts of cell lysates were subjected to immunoprecipitation with anti-EpoR, anti-JAK2, or NRS as indicated. In vitro kinase assays were performed as described in Materials and Methods. The phosphorylated proteins were resolved by 7.5% SDS-PAGE and detected by autoradiography. The positions of the tyrosine-phosphorylated EpoR, JAK2, and the 140-kD phosphorylated protein are indicated by arrows. (B) Secondary immunoprecipitation of the EpoR and JAK2 phosphorylated in vitro. The in vitro kinase reaction of the anti-EpoR immunoprecipitate from Epo-stimulated cells was performed as described above. The phosphorylated proteins were eluted with buffer containing SDS. Each quarter of the eluate was directly analyzed (1) or immunoprecipitated again with normal rabbit serum (2), anti-EpoR (3), or anti-JAK2 (4) before being subjected to SDS-PAGE and autoradiography. (C) Phosphoamino acid analysis of the proteins phosphorylated in immune complex kinase assays of anti-EpoR immunoprecipitates. EpoR (1), JAK2 (2), and the 140-kD protein (3) were subjected to phosphoamino acid analysis as described in Materials and Methods. The positions of migration of phosphoamino acid standards are indicated.
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Fig 4. Tyrosine phosphorylation of JAK2 and its association with EpoR in vivo correlate with the signal transducing abilities of mutant EpoRs. (A) Epo induces tyrosine phosphorylation of JAK2 in only the 32D clones expressing mitogenically active EpoRs. Parental 32D cells (Or), 32D/EpoR-Wt cells (Wt), and 32D transfectants expressing the H, PB, or PM4 mutants (H, PB, or PM4, respectively) were either left unstimulated (-) or were stimulated (+) for 5 minutes with Epo and solubilized. The cell lysates were used for immunoprecipitation with the 4G10 anti-phosphotyrosine monoclonal antibody. The positions of JAK2 and the tyrosine-phosphorylated wild-type EpoR are indicated. (B,C) JAK2 associates with only the mitogenically active EpoRs in vivo. The cell lysates were prepared as described above and used for immunoprecipitation with the anti-EpoR serum. The eluted samples were divided into two, resolved by SDS-PAGE, and transferred to two Immobilon-P membranes. The membranes were then probed with the 4G10 antiphosphotyrosine antibody (B) or anti-JAK2 serum (C).

These results support the hypothesis obtained from in vitro data that JAK2 associates with the membrane-proximal cytoplasmic region of the EpoR on Epo stimulation and couples Epo binding to tyrosine phosphorylation and mitogenesis.

The association of JAK2 with the EpoR was dependent on Epo stimulation. Previously, JAK2 was shown to bind in vitro with a recombinant fusion protein containing the cytoplasmic domain of the EpoR or bind in vivo with EpoR produced in insect cells. The JAK2 binding in both situations was independent of Epo stimulation. However, it is possible that the high concentrations of protein that are obtained in these experiments may not detect subtle changes in affinities of association that might occur in vivo under the concentrations and conditions associated with mitogenesis. Consistent with our in vivo binding results with EpoR, the association of JAK2 in vivo with the growth hormone receptor, another member of the cytokines receptor superfamily, requires ligand binding. However, JAK2 constitutively associates with the receptor for prolactin in vivo as well as the gp130 signaling subunit in the receptors for ciliary neurotropic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and IL-6.

The exact mechanisms by which Epo binding causes a prominent increase in affinity of the EpoR for JAK2 and activation of JAK2 kinase activity are still unknown. Homodimerization has been strongly implicated in the activation of the EpoR by a point mutation, Arg129 to Cys, that results in disulfide-linked homo-dimerization and constitutive activation of mitogenic activity. This mutation is associated with the constitutive, ligand independent, activation of JAK2 in vitro kinase activity (data not shown). Thus, it is possible that ligand-induced dimerization or oligomerization of the EpoR may be required for the binding of JAK2. The binding with EpoR might then alter the conformation of JAK2 to activate its kinase activity. Alternatively, the binding with

DISCUSSION

The present studies show that JAK2 associates with the EpoR after Epo stimulation, that activated JAK2 is the major tyrosine kinase activity that associated with EpoR and activation correlates with the abilities of mutant EpoRs to transduce a mitogenic signal. In particular, JAK2 failed to associate with two mutant receptors with an internal deletion and a point mutation, Trp342 to Arg, within the membrane-proximal cytoplasmic region (PB and PM4 mutants, respectively). These two mutants are completely inactive either induction of tyrosine phosphorylation or mitogenesis,11,18
each EpoR molecule in an oligomeric complex may result in dimerization of JAK2 and activation of its kinase activity. Activation of kinase activity by dimerization has been similarly proposed for several tyrosine-kinase receptors.41

JAK2 associates with the mitogenically active carboxyl-truncated H mutant but failed to associate with the inactivated PB or PM4 mutant. Thus, among these mutants, the binding of JAK2 correlated with the induction of tyrosine phosphorylation of JAK2, the activation of its kinase activity32 and mitogenesis.15,16 In our previous studies, JAK2 was found to associate with the recombiant cytoplasmic domain of PM4 mutant in vitro.27 However, it is likely that the point mutation did not lower the affinity of JAK2 binding sufficiently to eliminate physical interaction in vitro at high protein concentrations.

The immune complex kinase assays of the EpoR immunoprecipitates suggested that JAK2 is the major tyrosine kinase that physically associates with the EpoR. In addition, a 150-kD protein was associated with the EpoR and became tyrosine phosphorylated in vitro. A double immunoprecipitation experiment, using antiserum against JAK1, showed that this protein is not JAK1 (data not shown), although JAK1 becomes weakly tyrosine phosphorylated in vivo on Epo stimulation.27 The immune complex kinase assays also showed that the EpoR became tyrosine phosphorylated in vitro in the anti-JAK2 immunoprecipitates as well as in the anti-EpoR immunoprecipitates, thus suggesting JAK2 is the tyrosine kinase that phosphorylates the EpoR. Consistent with this hypothesis, coinfection of insect cells with baculoviruses expressing EpoR and JAK2 results in the tyrosine phosphorylation of EpoR.28

Linnekin et al26 showed tyrosine kinase activity associated with EpoR in Ba/F3 cells transfected with the receptor. The predominant protein phosphorylated in vitro was 97 kD, which was also found to bind ATP, thus raising the possibility that it may be a tyrosine kinase. However, though modulated by Epo stimulation, the tyrosine kinase activity was constitutively found in the EpoR immunoprecipitates and EpoR was not phosphorylated in vitro. Thus, the functional significance of this kinase activity is not obvious. Although a 92-kDa tyrosine-phosphorylated protein was found to associate with EpoR 10 to 30 minutes after Epo stimulation (Fig 2), in repeated experiments, we have not observed a 97-kD protein phosphorylated in vitro. It should be noted that repeated efforts to show that this substrate is c-fes have failed (data not shown). The differences in results may reflect the differences in cell lines or reagents used in these studies. However, it should be noted that Linnekin et al26 also observed a minor, 130-kD phosphorylated protein in the EpoR immune complex kinase assays, which may correspond to JAK2. Yoshimura and Lodish37 also showed that protein kinase activities associate with the EpoR. Using a thioleavable chemical cross-linker, they showed that a number of proteins in the cross-linked EpoR complex were phosphorylated in vitro. Unfortunately, it could not be addressed whether in vitro phosphorylation of these proteins was modulated by Epo stimulation. Among these proteins, the EpoR and a 130-kD protein that was cross-linked with the receptor reacted with antiphosphotyrosine antibody. Thus, it is possible that the 130-kDa protein was JAK2.

A limited number of tyrosine kinases including Lyn, Tec, Fes, JAK1, and JAK2 are expressed in IL-3-dependent hematopoietic cells.13 Of these kinases, Fes has been implicated in the signal transduction from the receptors for Epo,44 IL-3, and GM-CSF26 because these cytokines induced tyrosine phosphorylation of Fes and enhanced its kinase activity in a human erythroleukemia cell line, TF-1. However, in a variety of IL-3-dependent cell lines we have tested, neither IL-3 nor Epo showed any effect on tyrosine phosphorylation or kinase activity of Fes or of any other tyrosine kinases expressed except for JAK1 and JAK2.27 More specifically, we also have not detected changes in phosphorylation or kinase activity of Fes in TF-1 cells,28 whereas JAK2 is activated in the response of these cells to either Epo, GM-CSF, or IL-3. These observations are consistent with the hypothesis that JAK2 plays a major role in the growth signal transduction from the EpoR.

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Erythropoietin induces association of the JAK2 protein tyrosine kinase with the erythropoietin receptor in vivo

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