Induction of Tyrosine Phosphorylation of Vav and Expression of Pim-1 Correlates With Jak2-Mediated Growth Signaling From the Erythropoietin Receptor

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The receptor for erythropoietin (Epo) belongs to the cytokine receptor family and lacks a tyrosine kinase domain. However, it has been hypothesized that a tyrosine kinase, Jak2, associates with the membrane proximal cytoplasmic region of Epo receptor (EpoR) and mediates the growth signaling from the receptor through tyrosine phosphorylation of cellular substrates. To explore the growth signaling pathways from the EpoR, we analyzed substrates of tyrosine phosphorylation induced by Epo stimulation in cells expressing various mutant EpoRs. The vav proto-oncogene product was found to be tyrosine phosphorylated after Epo stimulation in cells expressing the wild-type EpoR or a truncated receptor, H mutant, that retains the growth signaling function. In these cells, Epo also induced the expression of a serine/threonine kinase, Pim-1. However, Epo stimulation did not have any effect on Vav or Pim-1 in cells expressing a mutant EpoR, PM4 mutant, inactivated by a point mutation, Trp282 to Arg, in the membrane proximal region, which abrogates the interaction with Jak2. On the other hand, both tyrosine phosphorylation of Vav and expression of Pim-1 were observed constitutively in cells expressing a mutant EpoR that is constitutively activated by a point mutation, Arg129 to Cys, in the extracellular domain. Jak2 was also constitutively tyrosine phosphorylated and activated in cells expressing this mutant, which confirms the crucial role of Jak2 in growth signaling from the EpoR. Taken together, these observations suggest that the tyrosine phosphorylation of Vav and the expression of Pim-1 may play important roles in growth signaling from the EpoR.

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MATERIALS AND METHODS

Cells and reagents. A clone of interleukin-3 (IL-3)—dependent 32D cell line and 32D clones expressing the wild-type or H- and PM4 mutant EpoRs were previously described. IL-3—dependent DA3 cells expressing the wild-type or a constitutively activated mutant EpoR were also previously described. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10% WEHI-3 conditioned medium as a source of IL-3. Rabbit polyclonal antiserum against the cytoplasmic portion of recombinant murine EpoR or against a synthetic peptide from Jak2 have been described. Antiphosphotyrosine monoclonal antibody (4G10) and rabbit antiserum against Vav and Pim-1 were purchased from Upstate Biotechnology, Inc (Lake Placid, NY). Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan).

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**Immunoprecipitation and antiphosphotyrosine immunoblotting.** For stimulation with Epo or IL-3, cells were cultured for 12 hours in RPMI medium without IL-3. The cells were then left unstimulated as a negative control or stimulated with a saturating concentration of Epo or IL-3 at 37°C for 10 minutes. The cells were lysed in a lysis buffer containing 1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 100 μmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL each of aprotinin and leupeptin.

For immunoprecipitation, cell lysates were incubated with a relevant antibody and protein A-Sepharose beads for 4 hours at 4°C. The immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes, probed with the 4G10 antiphosphotyrosine antibody, and developed by the enhanced chemiluminescence (ECL) system (Amersham Corp, Arlington Heights, IL). Aliquots of the cell lysates were also subjected to immunoblotting after directly mixed with equal volumes of 2× Laemmli’s sample buffer and heated at 100°C for 5 minutes. For re-probing with anti-Vav, the membranes were treated at 50°C for 30 minutes with stripping buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7) before incubation with anti-Vav.

**In vitro kinase assays.** Immunoprecipitates with anti-Jak2 antibody were prepared as described above and subjected to in vitro kinase assays as described previously.28 In brief, immunoprecipitates were incubated for 30 minutes at room temperature with kinase buffer (10 mmol/L HEPES, pH 7.4, 50 mmol/L NaCl, 5 mmol/L MgCl₂, 5 mmol/L MnCl₂, 100 μmol/L sodium orthovanadate) containing 250 μg/mL [γ-³²P] ATP. The proteins were resolved by SDS-PAGE and subjected to autoradiography.

**Immunoblot analysis of Pim-1 expression.** To examine the Pim-1 expression, cells were washed out of IL-3 for 12 hours and incubated in RPMI medium without growth factor or in RPMI medium supplemented with Epo or IL-3 for 4 hours at 37°C. After the incubation, the cells were directly lysed with 1× Laemmli’s SDS sample buffer and the lysates, equivalent from 5 × 10⁶ cells, were subjected to 12% SDS-PAGE followed by immunoblotting with anti-Pim-1.

**RESULTS**

Epo or IL-3 induces tyrosine phosphorylation of Vav in IL-3-dependent cells expressing the transfected EpoR. To examine substrates of tyrosine phosphorylation induced by stimulation with Epo or IL-3, an IL-3-dependent 32D cell line, 32D/EpoR-Wt, expressing the wild-type murine EpoR, was stimulated with Epo or IL-3 and the total cell lysates or anti-Vav immunoprecipitates were examined for tyrosine-phosphorylated proteins by antiphosphotyrosine immunoblotting. As shown in Fig 1, both Epo and IL-3 induced tyrosine phosphorylation of Jak2 (p130) and Shc (p52 and p49), whereas Epo specifically induced tyrosine phosphorylation of the EpoR (p72), in accordance with our previous studies.16,17 Unidentified major substrates of 92 kD and 70 kD (p92 and p70, respectively) were also observed after Epo or IL-3 stimulation, as previously described.15 In addition, antiphosphotyrosine blotting of the lysates from Epo- or IL-3-stimulated cells showed a broad band of tyrosine-phosphorylated protein(s) just above p92. Antiphosphotyrosine blotting of the anti-Vav immunoprecipitates showed that Epo and IL-3 comparably induce tyrosine phosphorylation of Vav. The tyrosine-phosphorylated Vav appeared as a 97-kD protein and should be contained in the broad band observed in the total cell lysates.

Epo-induced tyrosine phosphorylation of Vav was then examined in 32D clones expressing mutant EpoR variants previously described.11,12 32D/EpoR-H cells express the truncated H-mutant that lacks the carboxyl-terminal 108 amino acids, including tyrosine phosphorylation sites. However, this mutant retains the ability to couple with the Jak2 kinase to transduce a mitogenic signal.9,10 32D/EpoR-PM4 cells express the PM4 mutant, which has a mutation, Trp182 to Arg (W282R), in the membrane proximal cytoplasmic region showing a homology with other cytokine receptors.11 This mutation was shown to abolish the ability of EpoR to couple with Jak2.19 These clones were stimulated with Epo or IL-3 and the tyrosine phosphorylation of Vav was examined by antiphosphotyrosine blotting of anti-Vav immunoprecipitates. Figure 2 shows that Epo induces the tyrosine phosphorylation of Vav comparably with IL-3 stimulation in 32D/EpoR-H, whereas Epo failed to induce any detectable tyrosine phosphorylation of Vav in 32D/EpoR-PM4. Therefore, the carboxyl-terminal 108 amino acid region of EpoR was not required for induction of the tyrosine phosphorylation of Vav, whereas the membrane-proximal region required for coupling with Jak2 was also crucial for the Vav phosphorylation.

A constitutively activated EpoR mutant is associated with constitutive activation of Jak2 and tyrosine phosphorylation of Vav. A single point mutation, replacing Arg528 with Cys, has been shown to activate the receptor, independent of Epo binding.18 To explore the mechanisms of growth signaling from the activated mutant, we examined the status of Jak2 and Vav in a factor-independent clone, DA3/EpoR-R129C, obtained by transfecting the R129C mutant into an IL-3-dependent cell line, DA3.32 When Jak2 was immunoprecipitated from DA3/EpoR-R129C, in vitro kinase activity of Jak2 was detected in cells grown in the absence of growth factors (Fig 3). In contrast, no significant Jak2 in vitro kinase activity was detected in cells expressing the wild-type receptor, DA3/EpoR-Wt, when removed from growth factors, in accordance with our previous observation.11 Stimulation of DA3/EpoR-R129C cells with Epo resulted in a twofold to threefold increase in in vitro kinase activity. These results show that the constitutively activated R129C mutant causes ligand-independent activation of Jak2, which agrees with the hypothesis that Jak2 plays a crucial role in the EpoR-mediated growth signaling.

The status of tyrosine phosphorylation of Jak2, EpoR, and Vav in cells expressing the constitutively activated EpoR was then examined by antiphosphotyrosine blotting of immunoprecipitates obtained with relevant antibodies. As shown in Fig 4A, Jak2 was constitutively tyrosine phosphorylated in DA3/EpoR-R129C, whereas tyrosine phosphorylation of the mutant receptor itself was dependent on Epo stimulation. In DA3/EpoR-R129C cells, the tyrosine phosphorylation of Vav was observed without stimulation with growth factors and thus correlated with the activation of Jak2 (Fig 4B).

**Induction of Pim-1 expression by Epo in cells expressing the wild-type or mutant EpoRs.** The Pim-1 serine/threonine
kinase has been implicated in growth regulation of hematopoietic cells. To examine possible involvement of the Pim-1 kinase in the EpoR-mediated growth signaling, we analyzed the expression of Pim-1 in cells stimulated with Epo. After removal from IL-3 for 12 hours, 32D/EpoR-Wt cells were cultured for 4 hours in complete RPMI medium without growth factors or in medium supplemented with Epo or IL-3. The cells were then lysed and the expression of Pim-1 was analyzed by immunoblotting with anti-Pim-1.

Figure 5 (left panel) shows that Epo as well as IL-3 induced expression of the 33-kD Pim-1 kinase in 32D/EpoR. Epo-induced expression of Pim-1 was also examined in cells expressing the various mutant EpoRs. The truncated H mutant retained the ability to induce the Pim-1 expression, whereas the PM4 mutant with the inactivating mutation in the membrane proximal region was without this function (Fig 5, left panel). In cells expressing the constitutively activated R129C mutant, Pim-1 was expressed constitutively. The abilities of these mutant EpoRs to induce the Pim-1 expression thus correlated with their abilities to activate Jak2 and to induce proliferation of cells.

DISCUSSION

The present studies showed that Epo stimulation induces the tyrosine phosphorylation of Vav and the expression of Pim-1. Studies using clones expressing mutant EpoRs showed that the membrane proximal cytoplasmic region of the EpoR, previously shown to be crucial for activation of Jak2, is required for induction of both tyrosine phosphorylation of Vav and expression of Pim-1, whereas the carboxy-terminal 108 amino acid region is not required. Furthermore, Jak2 was shown to be constitutively activated in cells expressing the constitutively activated R129C mutant EpoR, in which both tyrosine phosphorylation of Vav and expression of Pim-1 were also constitutive. The effects of Epo on Vav and Pim-1 thus correlate with activation of Jak2 and induction of proliferation by these mutant EpoRs, which suggest that Vav and Pim-1 may play important roles in growth signaling from the EpoR.

Recent studies have shown that the vav proto-oncogene product, selectively expressed in hematopoietic cells,
Fig 3. Constitutive in vitro activity of Jak2 in cells expressing a constitutively activated mutant EpoR. Clones of DA3 cells expressing the wild-type EpoR (WT) or an EpoR mutant containing an Arg129 to Cys mutation (R129C) were treated with no growth factor (−) or with Epo (+). After stimulation, cells were lysed and Jak2 was isolated by immunoprecipitation with anti-Jak2 sera and subjected to an in vitro kinase assay. The products of the assay were separated by SDS-PAGE, transferred to nitrocellulose and detected by autoradiography (A). The blot was subsequently probed with anti-Jak2 sera (B).

Fig 4. Tyrosine phosphorylation of Jak2, EpoR, and Vav in cells expressing the constitutively activated mutant EpoR. (A) DA3 cells expressing the wild-type EpoR (WT) or the activated mutant (R129C) were left unstimulated or stimulated with Epo (Ep) or IL-3 (IL3) for 10 minutes as indicated before solubilization. The cell lysates were immunoprecipitated with anti-Jak2 (upper panel) or anti-EpoR (lower panel) and subjected to antiphosphotyrosine blotting. (B) The cells were treated in the same way as in (A). The cell lysates were immunoprecipitated with anti-Vav and subjected to antiphosphotyrosine blotting (αPY) followed by reprobing with anti-Vav (αVav).

1. Constitutive in vitro activity of Jak2 in cells expressing a constitutively activated mutant EpoR. Clones of DA3 cells expressing the wild-type EpoR (WT) or an EpoR mutant containing an Arg129 to Cys mutation (R129C) were treated with no growth factor (−) or with Epo (+). After stimulation, cells were lysed and Jak2 was isolated by immunoprecipitation with anti-Jak2 sera and subjected to an in vitro kinase assay. The products of the assay were separated by SDS-PAGE, transferred to nitrocellulose and detected by autoradiography (A). The blot was subsequently probed with anti-Jak2 sera (B).

Vav was shown in the present studies failed to correlate with activation of the MAP kinases, which required the carboxyl-terminal region of the EpoR and correlated with tyrosine phosphorylation of Shc and its association with Grb2. Vav may thus play a role in signaling pathways other than the Ras/MAP kinase pathways. It should be noted in this regard that Vav has several putative motifs seen in transcription factors as well as one Src homology 2 domain and two Src homology 3 domains, which are shared by many signaling adapter molecules.

The pim-1 gene was originally identified as a common site for retroviral insertion in T-cell lymphomas in mice. Previous studies showed that Epo also activates Ras, and we recently found that Epo stimulates activation of the MAP kinases. The tyrosine phosphorylation of Vav induced by Epo stimulation may thus play a role in activation of the Ras signaling pathways by the activated EpoR. However, inconsistent with this possibility, the tyrosine phosphorylation of Vav in cells expressing various mutant EpoRs rapidly and transiently tyrosine phosphorylated in response to stimulation of diverse hematopoietic cell receptors, including the T-cell receptor, the IgM receptor, the c-kit receptor, the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, and the IL-3 receptor. Sharing homology with guanine nucleotide releasing factors (GNRFs) that activate Ras-related small guanosine triphosphate (GTP)-binding proteins, Vav was shown to possess GNRF activity that increased after the T-cell receptor-CD3 triggering in T cells in parallel with its tyrosine phosphorylation. Furthermore, vav-transfected fibroblasts displayed constitutive activation of Ras and MAP kinases, known downstream intermediates in Ras-dependent signaling pathways. These observations suggest that Vav may play a role in activation of the Ras signaling pathways by hematopoietic cell receptors.

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Vav AND Pim-1 IN EPO SIGNALING

Subsequent studies showed that the *pim-1* gene is also involved in the generation of B-cell lymphomas and erythroleukemias. The product of the *pim-1* gene is a cytoplasmic serine/threonine kinase, expression of which is largely restricted to hematopoietic cells and is highly inducible by hematopoietic growth factors, including IL-2, IL-3, and GM-CSF. Furthermore, bone marrow-derived mast cells from Pim-1–deficient mice showed an impaired response to IL-3 in vitro. These observations suggest that Pim-1 may play a role in growth control of hematopoietic cells. In line with this hypothesis, the present studies showed that Epo also induces the expression of Pim-1, which correlated with the proliferation signal transduced from various mutant EpoRs. A possible involvement of Pim-1 in erythropoiesis in vivo was also suggested by a previous study showing a correlation between the Epo expression and the erythrocyte mean cell volume in Pim-1–deficient or Eμ–*pim-1* transgenic mice. Interestingly, a recent study on Eμ–*pim-1* transgenic mice suggested that Pim-1 may act as an inhibitor of apoptosis. Because one of the important functions of Epo is to prevent apoptosis of erythroid progenitor cells, it will be of interest to study a possible involvement of Pim-1 in EpoR-mediated prevention of apoptosis.

In cells expressing the constitutively activated mutant EpoR, Jak2 was constitutively tyrosine phosphorylated and displayed the autophosphorylation activity in vitro without Epo stimulation. This finding is consistent with the hypothesis that Jak2 is the kinase that mediates the growth signaling from the EpoR. In addition, these results suggest that the constitutively activated EpoR, which may be activated by dimerization, activates the same growth signaling pathway with the ligand-activated EpoR. It will be important to determine whether the activation of EpoR by the gp55 glycoprotein of the Friend spleen focus-forming virus, which is involved in the initial stage of Friend erythroleukemia, is also associated with activation of Jak2.

The tyrosine phosphorylation of the R129C mutant, which constitutively activates Jak2, was dependent on Epo stimulation. This finding is inconsistent with our previous studies, which suggested that the EpoR is a substrate for Jak2. However, it is possible that the tyrosine-phosphorylated EpoR may be susceptible to dephosphorylation by HCP, which binds the tyrosine phosphorylated carboxyl-terminal region of the EpoR (Yi et al, submitted) or other protein tyrosine phosphatases. Then, further activation of Jak2 by Epo stimulation may be required to cause easily detectable tyrosine phosphorylation of the R129C mutant. In contrast, there was very little enhancement of the phosphorylation of Vav after Epo stimulation in DA3/EpoR-R129C. It is thus suggested that the level of Jak2 kinase activity constitutively activated by the R129C mutant may be sufficient for causing, directly or indirectly, significant tyrosine phosphorylation of Vav. It is also implied that the tyrosine phosphorylated Vav may not be susceptible to dephosphorylation by protein tyrosine phosphatases.

The present studies showed that the membrane proximal cytoplasmic region of EpoR is required for induction of the expression of Pim-1. Because the membrane proximal region of EpoR is also required for association with and activation of Jak2, activation of Jak2 may be required for induction of Pim-1. Consistent with this possibility, recent studies using mutant βc chains of the GM-CSF receptor showed that the membrane proximal region of the βc chain is required for both Jak2 activation and induction of the Pim-1 expression. Similar to the results of our previous studies on EpoR mutants, the membrane distal region of the βc chain was dispensable for growth signaling but was required for the tyrosine phosphorylation of Shc and activation of the Ras/MAP kinase pathways by the GM-CSF receptor. Thus, tyrosine kinases other than Jak2 may be involved in activation of these pathways by the cytokine receptors. On the other hand, the tyrosine phosphorylation of Vav was apparently dependent on the membrane proximal region of the EpoR, thus suggesting that Jak2 may be directly responsible for its phosphorylation. Alternatively, activated Jak2 may activate a tyrosine kinase that may phosphorylate Vav. Irrespective, further studies toward identification of cellular substrates of Jak2 are required to elucidate the molecular and biochemical mechanisms of EpoR-mediated signaling.

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