RAPID COMMUNICATION

Erythropoietin Induces Tyrosine Phosphorylation and Kinase Activity of the c-fps/fes Proto-Oncogene Product in Human Erythropoietin-Responsive Cells

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Erythropoietin (EPO) is a hematopoietic growth factor that stimulates the proliferation and differentiation of erythroid progenitor cells. Although the EPO receptor has no kinase domain, EPO rapidly induces tyrosine phosphorylation of several proteins in EPO-responsive cells. Therefore, the receptor activation by the ligand could induce tyrosine-kinase activity of unidentified cellular protein(s). Here we show that c-fps/fes proto-oncogene product (p92"c-fes"), nonreceptor tyrosine kinase, is tyrosine-phosphorylated on treatment with EPO in a human erythroleukemia cell line TF-1 that is responsive to granulocyte-macrophage colony-stimulating factor, interleukin-3, and EPO. In addition, the kinase activity of p92"c-fes" was shown to be enhanced by treatment with EPO. Therefore, p92"c-fes" could be implicated in a signaling pathway triggered by EPO in human EPO-responsive cells.

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Preparation of cell lysates. Cells were incubated without FCS or growth factors for 8 to 15 hours before the experiment and then resuspended in RPMI medium 1640 containing 100 mmol/L Na2VO4. Cells were treated with 20 U/mL EPO for 5 minutes at 37°C. Cells were lysed at 4°C in the buffer containing 20 mmol/L Tris-HCl pH 8.0, 1% Nonidet P-40 (NP-40), 500 U/mL aprotinin, 2 mmol/L EDTA, 50 mmol/L NaF, and 1 mmol/L Na3V04. Unsolubilized materials were removed by centrifugation for 10 minutes at 15,000g at 4°C.

Immunoprecipitation. Cell lysates were mixed with polyclonal anti-p92"c-fes" antibody, or FI15 antibody (rat IgM) and a secondary rabbit polyclonal anti-rat IgM (Fc) (Nordic, Tilburg, The Netherlands). The immunoprecipitates were collected with protein A-Sepharose (Sigma, St Louis, MO). All the immunoprecipitates were intensively washed with the lysis buffer before resuspension in Laemmli's sample buffer.

Western blotting. Samples were subjected to 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) filters (Millipore, Waters, MA). Filters were blocked with the buffer containing 10 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 10% skim milk, and 0.05% Triton X-100 (Sigma). For Western blotting for phosphotyrosine-containing proteins, filters were incubated with PY20 antibody and then with goat alkaline-phosphatase-conjugated antimouse IgG (Fc) (Promega, Madison, WI). For Western blotting for p92"c-fes", filters were sequentially incubated with FI15 antibody (rat IgM), rabbit antirat IgM (Fc) antibody (Nordic), and goat alkaline-phosphatase-conjugated antirabbit IgG (Fc) antibody (Promega). After each incubation, filters were washed four times in the buffer containing 10 mmol/L Tris-HCl pH 8.0, 150

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mmol/L NaCl, and 0.05% Triton X-100. Color reaction was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega).

**Kinase assays.** Cell lysates were mixed with polyclonal anti-p92Fes antibody, and the immune complexes were collected with protein A-sepharose and suspended in kinase buffer (25 mmol/L HEPES pH 7.5, 0.1% NP-40, and 3 mmol/L MnCl₂). After addition of 10 μCi[γ-32P] adenosine triphosphate (ATP), the mixture was incubated for 15 minutes at room temperature and subjected to 7% SDS-PAGE. Phosphorylated proteins were detected by Fujix BAS 2000 Bio-image Analyzer (Fuji Film, Tokyo, Japan).

### RESULTS

**Expression of p92Fes in TF-1.** TF-1 is a human erythroleukemia cell line that requires GM-CSF or IL-3 for growth, and the cells die within several days when the factors are depleted even in medium supplemented with FCS. EPO also supports the short-term growth of TF-1 cells for no more than 10 days. To check the expression of p92Fes in TF-1 cells, total lysates of TF-1 cells were immunoblotted with F115 antibody (Fig 1). p92Fes was expressed in TF-1 cells, although, as a negative control, it was not expressed in a human choriocarcinoma cell line BeWo. p92Fes was also expressed in other human EPO-responsive cells including UT-7 and F36E cells (data not shown). The amount of p92Fes was not affected by treatment with EPO in TF-1 cells. Although a distinct 94-Kd protein (p94Fes), antigenically related to p92Fes, has been identified in a number of hematopoietic and nonhematopoietic human cells, F115 antibody did not recognize p94Fes (data not shown).

**EPO-induced tyrosine phosphorylation of p92Fes.** To determine whether p92Fes is tyrosine-phosphorylated by treatment with EPO, we immunologically purified p92Fes with polyclonal anti-p92Fes antibody and tested the phosphorylation level of the protein by Western blotting with PY20 antibody. p92Fes was shown to be tyrosine-phosphorylated by treatment with EPO (Fig 2). This result shows that EPO induces tyrosine phosphorylation of p92Fes in TF-1 cells.

**EPO-induced kinase activity of p92Fes.** We then evaluated the effect of EPO on the kinase activity of p92Fes in TF-1 cells. The factor-starved cells were incubated for 5 minutes in the presence or absence of EPO. The cell lysates were
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Fig 3. EPO enhances the kinase activity of p92"" in TF-1 cells. Lysates of untreated (lane 1) or EPO-treated (lanes 2 and 3) TF-1 cells (1 × 10^7 cells/lane) were mixed with polyclonal anti-p92"" antibody (lanes 1 and 2) or normal rabbit serum (lane 3). The immunoprecipitates were subjected to in vitro kinase assay. The arrow indicates the position of p92"".

subjected to immunoprecipitation with polyclonal anti-p92"" antibody and the in vitro immune-complex kinase assay detected the enhanced level of phosphorylation of p92"" by treatment with EPO (Fig 3). This result shows that EPO enhances the kinase activity of p92"" in TF-1 cells.

DISCUSSION

A number of investigators have shown that several proteins are rapidly tyrosine-phosphorylated on treatment with EPO in EPO-responsive cells. It has already been reported that EPO induces tyrosine phosphorylation of guanosine triphosphatase (GTPase)-activating protein (GAP) at the molecular weight of 120 Kd in a human erythroleukemia cell line HEL, and that EPO also induces tyrosine phosphorylation of its own receptor at the molecular weight of 75 Kd in UT-7 cells. The other tyrosine-phosphorylated proteins have not been identified. Here we have shown that EPO induces tyrosine phosphorylation of p92"". We also have shown that EPO enhances the kinase activity of p92"". So far, some investigators have noted that EPO induces tyrosine phosphorylation of a protein, or proteins, at the molecular weights of 90 to 100 Kd. However, many researchers failed to show that EPO induces tyrosine phosphorylation of p92"", probably because of the low quality of anti-p92"" antibodies or the poor responsiveness of cells to EPO.

The expression of p92"" at relatively high levels is restricted to GM progenitor cells and p92"" is a possible candidate for tyrosine kinases involved in the signal transduction of GM-CSF and IL-3. However, p92"" is also expressed in EPO-responsive cells including TF-1, UT-7, HEL cells that are considered to originate from stem cells capable of differentiating into erythroid cells.

The expression of the cytoplasmic domain of the EPO receptor in IL-3-dependent cells downmodulates proliferative responsiveness to GM-CSF, suggesting that GM-CSF and EPO might share a part of components in their signaling pathways. It has also been suggested that the EPO and IL-3 receptors are capable of using the same tyrosine kinase in IL-3-dependent cells transfected with the EPO receptor, because IL-3-dependent cells become EPO-dependent when the cDNA encoding the EPO receptor alone is transfected into the cells and EPO induces almost the same pattern of tyrosine phosphorylation in these cells. From these observations combined with our results, it is possible that p92"" is implicated in a signaling pathway triggered by EPO as well as GM-CSF and IL-3 in cells having potential multilineage phenotypes as seen in TF-1 cells.

Recently, it has been reported that the EPO receptor is associated with tyrosine kinase of a 97-Kd phosphotyrosine-containing protein. Whether the EPO receptor is associated with p92"" has not been examined in this study because anti-EPO receptor antibodies are not yet available in our laboratory. However, the EPO receptor is reported to be composed of multi-subunit complex. It is also possible that p92"" is associated with an unidentified second subunit. Protein(s) interacting with p92"" and precise physiologic roles of p92"" in the signal transduction of EPO remains to be further elucidated.

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