ERYTHROPOIETIN (EPO) is a growth factor controlling erythropoiesis and is already widely applied for a variety of clinical use.1 EPO binds to a cell-surface receptor that has been molecularly cloned.2 The EPO receptor is a member of the cytokine receptor family and does not have any known catalytic domains.3 Molecular events that are activated after the binding of EPO to cell-surface receptors are still unknown. However, it has been shown that some tyrosine-kinase activation is crucial in transmitting the intracellular mitogenic signals triggered by binding of ligands to their receptors that do not contain any kinase domains. For instance, CD4/CD8, T-cell antigen receptor,7 surface IgM,8 and interleukin-2 (IL-2) receptor9 are associated with the src-family protein-tyrosine kinases, p56∗∗, p59 src, p56, and p56, respectively, and these tyrosine kinases are considered to be involved in their signal transduction. EPO has also been reported to induce rapid tyrosine-phosphorylation of some cellular proteins including its own receptor.10,16 Therefore, tyrosine-kinase activity of unidentified cellular protein(s) should be involved in a signaling pathway triggered by EPO. We report here that EPO induces tyrosine phosphorylation and kinase activity of c-fps/fes proto-oncogene product (p92∗∗∗), nonreceptor tyrosine kinase, in a human erythroleukemia cell line TF-1.

MATERIALS AND METHODS

Cell lines and growth factors. TF-1 cells were maintained in RPMI medium 1640 containing 10% fetal calf serum (FCS) and 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF).17 BeWo cells were obtained from the American Type Culture Collection (Rockville, MD) and they were maintained in Ham’s F-12 medium containing 10% FCS. Recombinant human GM-CSF and recombinant human EPO was generously provided by Schering Plough Co Ltd (Osaka, Japan) and Chugai Pharmaceutical Co Ltd (Tokyo, Japan), respectively.

Antibodies. Polyclonal anti-p92∗∗∗ antibody was prepared from sera of a rabbit immunized against a synthetic peptide that was conjugated with keyhole limpet hemocyanin (KLH) (Pierce, Rockford, IL). The peptide sequence was LLLQDDRHSTSSSEQERE-QGG corresponding to the amino acid residues of p92∗∗∗ 424-443, an upstream region of the SH2 domain.18 F115 antibody is a rat monoclonal anti-p92∗∗∗ antibody (Onogene Science, Manhasset, NY). F115 antibody recognizes a region within the kinase domain and it blocks the kinase activity.19 PY20 antibody is a mouse monoclonal antiphosphotyrosine antibody (ICN, Irvine, CA).

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 μmol/L Na3VO4. Cells were treated with 20 U/mL EPO for 5 minutes at 37°C. Cells were lysed by 4°C in the buffer containing 20 mmol/L Tris-HCl pH 8.0, 1% Nonidet P-40 (NP-40; Iwai Kagaku, Tokyo, Japan), 1 mmol/L phenylmethylsulfon fluoride (PMSF), 500 U/mL aprotnin, 2 mmol/L EDTA, 50 mmol/L NaF, and 1 mmol/L Na3VO4. Unsolubilized materials were removed by centrifugation for 10 minutes at 15,000g at 4°C.

Immunoprecipitation. Cell lysates were mixed with polyclonal anti-p92∗∗∗ antibody, or F115 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 electrotransferred 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) antibody (Promega, Madison, WI). For Western blotting for p92∗∗∗, filters were sequentially incubated with F115 antibody (rat IgM), rabbit antirat IgM (Fc) antibody (Nordic), and goat alkaline-phosphatase-conjugated antimouse 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 mmol/L NaCl, 0.05% Tween-20, and 50 mmol/L NaF.

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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-p92* 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 p92* in TF-1.** TF-1 is a human erythro-leukemia 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 p92* in TF-1 cells, total lysates of TF-I cells were immunoblotted with F115 antibody (Fig 1). p92* was expressed in TF-I cells, although, as a negative control, it was not expressed in a human choriocarcinoma cell line BeWo. p92* was also expressed in other human EPO-responsive cells including UT-7 and F36E cells (data not shown). The amount of p92* was not affected by treatment with EPO in TF-1 cells. Although a distinct 94-Kd protein (p94*), antigenically related to p92*, has been identified in a number of hematopoietic and nonhematopoietic human cells, F115 antibody did not recognize p94* (data not shown).

**EPO-induced tyrosine phosphorylation of p92*.** To determine whether p92* is tyrosine-phosphorylated by treatment with EPO, we immunologically purified p92* with polyclonal anti-p92* antibody and tested the phosphorylation level of the protein by Western blotting with PY20 antibody. p92* was shown to be tyrosine-phosphorylated by treatment with EPO (Fig 2). This result shows that EPO induces tyrosine phosphorylation of p92* in TF-1 cells.

**EPO-induced kinase activity of p92*.** We then evaluated the effect of EPO on the kinase activity of p92* 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 p92fes 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-p92fes 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 p92fes.

subjected to immunoprecipitation with polyclonal anti-p92fes antibody and the in vitro immune-complex kinase assay detected the enhanced level of phosphorylation of p92fes by treatment with EPO (Fig 3). This result shows that EPO enhances the kinase activity of p92fes 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 p92fes. We also have shown that EPO enhances the kinase activity of p92fes. 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 p92fes, probably because of the low quality of anti-p92fes antibodies or the poor responsiveness of cells to EPO.

The expression of p92fes at relatively high level is restricted to GM progenitor cells and p92fes is a possible candidate for tyrosine kinases involved in the signal transduction of GM-CSF and IL-3. However, p92fes is also expressed in EPO-responsive cells including TF-1, UT-7, F-36E, and 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 p92fes 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 p92fes 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 p92fes is associated with an unidentified second subunit. Protein(s) interacting with p92fes and precise physiologic roles of p92fes in the signal transduction of EPO remains to be further elucidated.

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