STAT5 Activation Correlates with Erythropoietin Receptor-mediated Erythroid Differentiation of an Erythroleukemia Cell Line*

(Received for publication, October 24, 1996, and in revised form, January 15, 1997)

Ken Iwatsuki‡§, Takaho Endo‡, Hiroyuki Misawa‡, Masahiro Yokouchi‡, Akira Matsumoto‡, Motoaki Ohtsubo‡, Kazuhiro J. Mori¶, and Akihiko Yoshimura‡

From the Institute of Life Science, Aikawamachi 2432-3 Kurume 839, Japan, the §Cellular Biochemistry, Animal Resource Science/Veterinary Medical Science, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, and the ¶Laboratory of Molecular and Cellular Science, Department of Biology, Faculty of Science, Niigata University, Niigata 950-21, Japan

Interaction between erythropoietin (EPO) and its membrane receptor induces the proliferation and differentiation of erythroid progenitors. EPO has been shown to activate the JAK2-STAT5 pathway in various hematopoietic cell lines, although the physiological role of this pathway is unclear. We have previously shown that epidermal growth factor activates a chimeric receptor bearing the extracellular domain of the epidermal growth factor receptor and the cytoplasmic domain of the EPO receptor, resulting in proliferation of interleukin-3-dependent hematopoietic cells and erythroid differentiation (globin synthesis) of EPO-responsive erythroleukemia cells. In the present study, we introduced various deletion and tyrosine to phenylalanine substitution in the cytoplasmic domain of the chimeric receptor and expressed these mutant chimeras in an EPO-responsive erythroleukemia cell line, ELM-I-1. Mutant chimeric receptors retaining either Tyr343 or Tyr401 could activate STAT5, judged by tyrosine-phosphorylation of STAT5 and induction of CBS, a target gene of STAT5. These mutants were able to induce erythroid differentiation. However, a chimeric receptor containing both Y343F and Y401F mutations could not activate STAT5 nor induce erythroid differentiation. Thus, Tyr343 or Tyr401 of the EPO receptor are independently necessary for erythroid differentiation as well as STAT5 activation. Moreover, exogenous expression of dominant-negative STAT5 suppressed EPO-dependent erythroid differentiation. These findings suggest that STAT5 plays an important role in erythroid differentiation through the EPO receptor cytoplasmic domain.

Erythropoietin (EPO) is a glycoprotein hormone required for the survival, proliferation, and differentiation of committed erythroid progenitor cells (1). The EPO receptor (EPOR) belongs to the cytokine receptor superfamily, which includes receptors for other hematopoietic growth factors such as interleukins, colony-stimulating factors, and growth hormone (2). A novel subfamily of protein tyrosine kinases, known as Janus kinases (JAKs), has been shown to associate with cytokine receptors, including the EPOR, and to play an important role in cytokine-dependent cell proliferation and gene regulation (3). Activated JAKs in turn convert latent cytoplasmic transcription factors, known as STATs (signal transducers and activators of transcription), into their active forms by tyrosine phosphorylation. The tyrosine phosphorylated STATs then translocate into the nucleus, where they bind to their specific target sequences, and then regulate expression of target genes (4).

The STAT family presently includes six members, each of which functions in a specific cytokine system. STAT5, which was originally identified as mammary gland factor regulated by prolactin (5), is activated by multiple cytokines such as IL2, IL3, IL5, granulocyte-macrophage colony stimulating factor, EPO, and thrombopoietin (6–11). Although IL2 and the other cytokines activate different JAKs, they all activate STAT5. The physiological function of STAT5 in hematopoietic cells, however, remains unclear. The role of STAT5 in cell proliferation is still controversial (9, 12–16), and little is known about its role in differentiation. However, because STAT3 has been shown to play a critical role in macrophage differentiation through gp130 (17–19), it would be interesting to determine whether STAT5 is involved in differentiation. We previously reported that exogenous expression of a chimeric receptor containing the extracellular domain of the EGF receptor (EGFR) and the cytoplasmic domain of the EPOR conferred EGF-dependent erythroid differentiation on EPO-responsive cells (20). Moreover, the membrane-proximal 127 amino acids are competent to induce differentiation. This region contains only one tyrosine residue (Tyr343), but it is sufficient to activate STAT5 (12–15). To clarify the role of STAT5 in the EPOR-mediated differentiation, we constructed various deletion and tyrosine substitution of chimeric EPOR mutants and introduced them into a highly EPO-responsive cell line, ELM-I-1. Tyrosine-mutant receptors that failed to activate STAT5 could not induce erythroid differentiation, suggesting that STAT5 positively regulates EPOR-mediated erythroid differentiation.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The wild type EGFR-EPOR chimera (designated wt chimera in this study) and a truncated chimeric receptor (EGFR-EPORH, designated –108 in this study) consisting of the extracellular domain of the EGFR and the cytoplasmic domain of the EPOR have been described previously (20, 21). These chimeric cDNAs were

*This was supported in part by grants from the Ministry of Science, Education and Culture of Japan, the Kato Memorial Foundation, the Japanese Foundation for Multidisciplinary Treatment of Cancer, the Haraguchi Memorial Foundation, the Uehara Memorial Foundation, the Kowa Life Science Foundation, and the Naito Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Inst. of Life Science, Kurume University, Aikawamachi 2432-3 Kurume 839, Japan. Tel.: 81-942-37-6313; Fax: 81-942-31-5212; E-mail: yoshimura@isl.kurume-u.ac.jp.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
subcloned into the mammalian expression vector pcDNA3 (Invitrogen) using EcoRI and XhoI sites. Delecion of -181 was created by adding stop codon and an hemagglutinin tag (20) at the BamHI site of the EPOR cytoplasmic domain, according to the procedure employed for -108 deletion. Deletion mutants -55, -76, and -135 were created by adding stop codon at the indicated position in the EPOR cytoplasmic domain by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers as follows. The forward primer, CCGCCGTACGCTGCAGCAGAGGACTCT (includes the Spl I site (21)). The reverse primers (include the EcoR I site and the stop codon), AGAATTCAGGGGTCCAGGATGGTGAGGC and reverse primer GGATCCAGGGGTCCAGGATGGTGAGGTG. The integrity of the sequences was confirmed by sequencing. The resulting constructs are designated Y2 (24), a hematopoietic cell-specific tyrosine phosphatase to Tyr219 (Y2) (25, 26), and SyP, another phosphatase to Tyr435 (Y2) (27). Although it has not been demonstrated that STAT5 directly binds to the phosphorylated tyrosine residues of the EPOR, Tyr433 (Y1) and Tyr435 (Y2) have been shown to be independently necessary for STAT5 activation. Thus, specific tyrosine residues of the EPOR are probably responsible for each signal transduction pathway. To clarify which tyrosine residues are important for EPOR-mediated erythroid differentiation, we constructed chimeric receptors (Fig. 1) bearing the extracellular domain and the transmembrane domain of the EGFR linked to full-length (wt) or several deletion mutants of the cytoplasmic domain of EPOR ( -55, -76YY, -108, -135Y, and -181 in Fig. 1). Previous reports have indicated that deletion of the 135 C-terminal amino acids can support proliferation in Ba/F3 cells but that the -181 mutant cannot (21, 28). Tyrosine substitution mutants were also created as shown in Fig. 1.

These chimeric receptors were introduced into erythroleukemia cell line ELM-I-1. Previously, we expressed wt and -108 chimeras in a Friend virus-transformed erythroleukemia cell line, TSA8, and found that these chimeras could induce erythroid differentiation (20). Whereas EPO induced globin synthesis or extrusion of the nucleus. Moreover, part of the endogenous EPOR may be activated by gp55 of the Friend virus (29). ELM-I-1, on the other hand, is retrovirus-free, and EPO induces not only globin but also hemoglobin synthesis (approximately 10–30% of cells become benzidine-positive after cultivation in the presence of EPO (30)). Thus, EPO can induce greater terminal

![Diagram](image)

**RESULTS AND DISCUSSION**

**Construction of Chimeric Receptors and Their Expression in ELM-I-1**—Phosphorylated tyrosine residues of the EPOR have been shown to be important for recruiting signal transducing molecules containing the SH2 domain. For example, phosphatidylinositol 3-kinase binds to phosphorylated Tyr219 (Y2) (25) in Fig. 1(24), a hematopoietic cell-specific tyrosine phosphatase to Tyr219 (Y2) (25, 26), and SyP, another phosphatase to Tyr435 (Y2) (27). Although it has not been demonstrated that STAT5 directly binds to the phosphorylated tyrosine residues of the EPOR, Tyr433 (Y1) and Tyr435 (Y2) have been shown to be independently necessary for STAT5 activation. Thus, specific tyrosine residues of the EPOR are probably responsible for each signal transduction pathway. To clarify which tyrosine residues are important for EPOR-mediated erythroid differentiation, we constructed chimeric receptors (Fig. 1) bearing the extracellular domain and the transmembrane domain of the EGFR linked to full-length (wt) or several deletion mutants of the cytoplasmic domain of EPOR ( -55, -76YY, -108, -135Y, and -181 in Fig. 1). Previous reports have indicated that deletion of the 135 C-terminal amino acids can support proliferation in Ba/F3 cells but that the -181 mutant cannot (21, 28). Tyrosine substitution mutants were also created as shown in Fig. 1.

These chimeric receptors were introduced into erythroleukemia cell line ELM-I-1. Previously, we expressed wt and -108 chimeras in a Friend virus-transformed erythroleukemia cell line, TSA8, and found that these chimeras could induce erythroid differentiation (20). Whereas EPO induced globin synthesis in TSA8 cells, EPO alone was incapable of inducing more mature differentiation, such as hemoglobin synthesis or extrusion of the nucleus. Moreover, part of the endogenous EPOR may be activated by gp55 of the Friend virus (29). ELM-I-1, on the other hand, is retrovirus-free, and EPO induces not only globin but also hemoglobin synthesis (approximately 10–30% of cells become benzidine-positive after cultivation in the presence of EPO (30)). Thus, EPO can induce greater terminal
erythroid differentiation in ELM-I-1 than in TSA8. In particular, IL3 strongly potentiates EPO-induced erythroid differentiation (30). This is quite similar to natural bone marrow cell differentiation in the presence of EPO and IL3. In several artificial systems, e.g. Ba/F3 sublines expressing exogenous EPOR (31, 32), IL3 suppressed EPO-induced globin synthesis. In this sense, ELM-I-1 cells seem to be more suitable for the study of erythroid differentiation in vitro than other EPO-dependent hematopoietic cells. We therefore introduced chimeric receptors into this cell line.

Differentiation Potential of Chimeric Receptor Mutants—The cDNAs for the chimeric receptors subcloned into a mammalian expression vector were introduced into the ELM-I-1 cells. Expression of the hybrid receptors was examined by flow cytometry using anti-EGFR antibody (data not shown). Positive clones (2–3 clones per chimeric receptor) were cultured in the absence (−) or presence of 10 unit/ml EPO (EPO) or 1 μg/ml EGF (EGF) for 3 days and then stained with benzidine. Representative photographs are shown.

Fig. 2. Benzidine staining of -76 tyrosine mutants. Each transformant (−76YY, −76YF, −76FY, and −76FF) was cultured in the absence (−) or presence of 10 unit/ml EPO (EPO) or 1 μg/ml EGF (EGF) for 3 days and then stained with benzidine. Representative photographs are shown.

Fig. 3. Globin content of transformants. Each transformant or parental ELM-I-1 (cont.) was cultured in the absence (−) or presence of 10 unit/ml EPO (EPO) or 1 μg/ml EGF (EGF) for 3 days, and the cellular content of globin polypeptide was detected by immunoblotting with anti-mouse globin.

Fig. 4. Activation of STAT5 by chimeric receptor mutants. A, transformants (1 × 10⁷/sample) were incubated in serum-free DMEM medium for 5 h at 37 °C and then stimulated with or without 1 μg/ml EGF at 25 °C for 10 min. Cell extracts were immunoprecipitated (IP) with anti-JAK2 (αJAK2) or anti-STAT5 (αSTAT5), then resolved by SDS-polyacrylamide gel electrophoresis, and then immunoblotted (blot) with anti-phosphotyrosine (αPY), αJAK2, or αSTAT5 antibodies. B, transformants were cultured in 1 μg/ml EGF or 10 unit/ml EPO for 2 h, and then total RNA (10 μg/lane) was separated from the cells and hybridized with CIS and α-enolase probes.

containing only two tyrosine residues (Y1 and Y2) (Fig. 1, −76YY). As shown in Figs. 2 and 3, the −76FY mutant, which contains Y343F substitution but retains Y1, was also capable of inducing EGF-dependent erythroid differentiation. Similarly, −76YF, which contains Y401F but retains Y1, was also capable of inducing differentiation (Figs. 2 and 3, −76YF). However, −76FF, which contains double mutation of Y343F and Y401F, could not induce EGF-dependent differentiation (Figs. 2 and 3, −76FF). Thus, one of the two tyrosine residues in the −76 deletion mutant is independently critical for erythroid differentiation.

JAK2 and STAT5 Activation by Tyrosine Mutants—To confirm that the −76FY and the −76YF mutant but not the −76FF mutant can activate STAT5 in ELM-I-1 cells, we measured STAT5 phosphorylation and CIS-induction through the mutant chimeric receptors. The −181, −76YY, −76YF, −76FY, and −76FF transformants were stimulated with EGF, and
then JAK2 and STAT5 were immunoprecipitated. Their tyrosine phosphorylation was detected with anti-phosphotyrosine antibody. As shown in Fig. 4, EGF stimulation in every mutant chimera. JAK2 phosphorylation was observed even in the –181 mutant, probably because this mutant contains box1 and box2 regions, which are putative JAK binding sites (Fig. 1). Thus, activation of JAK2 does not require tyrosine residues in the EPOR cytoplasmic domain. In contrast, STAT5 was not phosphorylated in response to EGF in the –181 nor the –76FF mutant, whereas –76YY, –76YF, and –76FY were able to induce STAT5 phosphorylation.

CIS is an immediate early gene induced by multiple cytokines such as IL2, IL3, and EPO in various hematopoietic cells and a direct target of STAT5 (12, 33). We prepared RNA from EGF- or EPO-stimulated cells expressing –76YY, –76YF, –76FY, and –76FF. Whereas EPO induced CIS expression in all transfamants, EGf induced CIS in –76YY, –76YF, and –76FY mutant cells but not in –76FF mutant cells (Fig. 4B). These data are consistent with previous reports that Y1 or Y2 of the EPOR cytoplasmic domain is sufficient to activate STAT5.

**Effect of Dominant-negative STAT5**—To clarify the critical role of STAT5 in erythroid differentiation, we expressed wt and dn type STAT5 in ELM-I-1 cells. The dominant-negative form of sheep STAT5 was created by deleting the C-terminal transactivation domain as reported by Mui et al. (16). This construct was shown to inhibit STAT5 activation and partially suppress cell proliferation. The Myc epitope tag was introduced into the N terminus to detect exogenous protein. Three independent clones from each transfection were tested for EPO-induced hemoglobin synthesis. As shown in Fig. 5, expression of wtSTAT5 did not affect EPO-induced benzidine positivity, whereas dnSTAT5 suppressed differentiation to about 5%. These data support that STAT5 plays a critical role in EPO-mediated erythroid differentiation in ELM-I-1 cells. However, we could not rule out the possibility that dnSTAT5 may suppress the activation of other signaling molecules through Y1 and Y2, because dnSTAT5 may tightly bind to these two phosphorylated tyrosine residues. Extensive study is under way to determine whether STAT5 is directly involved in differentiation.

Our results contrast strongly with a recent report by Chretien et al. (34). They found that EPO-induced differentiation in a human leukemia cell line, TF-1, correlates with impaired STAT5 activation, although direct evidence that STAT5 suppression was not caused by the difference in cell lines used. ELM-I-1 can grow in the absence of any cytokines, whereas TF-1 requires granulocyte-macrophage colony stimulating factor for growth. These two cells may be derived from different developmental stages of hematopoietic cells, and such differences may cause distinct STAT5 requirements for differentiation. However, the same as natural BFU-E cells, IL3 enhances EPO-induced erythroid differentiation of ELM-I-1 cells, although it does not induce erythroid-differentiation by itself. It is particularly important to examine the role of STAT5 in in vivo differentiation of natural erythroid progenitor cells. Studies are under way using bone marrow and fetal liver cells infected with retrovirus bearing chimeric receptors or dnSTAT5.

**Acknowledgments**—We thank Dr. H. Wakao for anti-STAT5 antiserum and H. Oghusu for technical assistance.

**REFERENCES**