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A Possible Involvement of Stat5 in Erythropoietin-Induced Hemoglobin Synthesis

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Erythropoietin (EPO) and its cell surface receptor (EPOR) play central roles in the proliferation and differentiation of mammalian erythroid progenitor cells. Recently both the tyrosine residues in the EPOR responsible for the activation of Stat5 and the role of Stat5 for EPO-dependent cell proliferation have been shown. Here, we describe the roles of Stat5 and of these tyrosine residues in the EPOR in the erythroid differentiation of murine hematopoietic cell line SKT6 which produces hemoglobin in response to EPO. Chimeric receptors carrying the extracellular domain of the EGF receptor and the intracellular domain of the EPOR were introduced into SKT6 cells. Like EPO, EGF equally activated Stat5 and induced hemoglobin. Activation of Stat5 and hemoglobin expression by EGF were markedly impaired in cells expressing the tyrosine mutated chimeric receptors. In addition, ectopic expression of the prolactin receptor, another cytokine receptor that activates Stat5, led to hemoglobin synthesis. Finally, hemoglobin synthesis was severely inhibited by overexpressing a dominant negative form of Stat5. These results collectively suggest that Stat5 plays a role in EPO-mediated hemoglobin synthesis in SKT6 cells.

Erythropoietin (EPO) is a lineage-restricted cytokine required for survival, proliferation, and differentiation of committed erythroid progenitor cells (1). EPO exerts its function through the EPO receptor (EPOR), a member of the class I cytokine receptor family.

It has been a subject of debate whether erythroid differentiation is absolutely dependent on specific signals emanating from the EPOR or not. When the EPOR is ectopically expressed in the IL-3-dependent cell line Ba/F3, EPO induces globin mRNA synthesis, while IL-3 suppresses EPO-induced globin expression (2-6). In contrast, erythroid differentiation of bone marrow cells from transgenic mice that express the human GM-CSF receptors has been observed without EPO(7). In this model, GM-CSF and probably IL-3 as well, can trigger EPO-like signals when its receptor is expressed in the erythroid lineage.

Structure-function analysis of the EPOR has defined the domains necessary for the activation of distinct signal. The membrane-proximal domain comprising box 1 and 2 is required for the induction of *c-myc, pim-1,* and *cis,* while the membrane distal region is necessary for the Ras/Raf-1/MAP kinase (MAPK) cascade, Syp, PLC-γ activation and for the induction of *c-fos* (8,9). The membrane proximal domain is also responsible for Jak2 and Stat5 activation, though the biological significance of this pathway in EPO signaling remains uncertain. Analysis of receptor tyrosine mutants defective in Stat5 activation well as the study of a dominant negative form of Stat5 have implicated Stat5 in cytokine-dependent cell proliferation (10-13).

In this study we have asked whether Stat5 and the critical tyrosine residues within the EPOR responsible for Stat5 activation play a role in erythroid differentiation using an EPO-responsive cell line, SKT6 (14). SKT6 cells were derived from the spleens of mice infected with anemic Friend spleen focus-forming virus and produce hemoglobin in response to EPO although they proliferate autonomously. We find herein that signals leading to hemoglobin synthesis emanating from the EPOR can be substituted with those from the prolactin (PRL) receptor, which also activates the Jak2-Stat5 pathway and that the critical tyrosine residues for Stat5 activation in the EPOR are indispensable for hemoglobin synthesis. Finally, the importance of Stat5 in EPO-induced hemoglobin synthesis is demonstrated by expressing a dominant negative form of Stat5.

MATERIALS AND METHODS

Cells. The murine SKT56 cells (generously provided by Dr. Todokoro, Riken, Japan) were cultured in RPMI 1640 medium containing

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7 % fetal bovine serum (FBS), 50 μM β-mercaptoethanol, and 50 μg/ml gentamycin. SKT6/EGFR-EPOR, EGFR-EPOR(Y343F), EGFR-EPOR(Null), SKT6/PRLR and STK6(Stat5Y694F) cells were cultured in the same medium plus G418 (0.5 mg/ml).

Plasmids. The cDNA for the rat PRL receptor (PRLR) (15) was introduced into pME18S. The cDNA for the chimeric receptor (EGFR-EPOR) consisting of the extracellular domain of the human EGFR and the cytoplasmic domains of the murine EPOR was provided by Dr. A. Yoshimura (Kurume Univ., Japan). The chimeric receptors EGFR-EPOR(NULL) and EGFR-EPOR(Y343F) were constructed with the PCR amplified extracellular domain of the human EGFR and the cytoplasmic domains of the mutant EPOR in pME18S (10). The sequences of the resulting receptors were verified by DNA sequencing.

Generation of stable transfectants. pME18S-Neo containing the PRLR or the EGFR-EPOR, the EGFR-EPOR(NULL), the EGFR-EPOR(Y343F) or ovine Stat5(Y694F) (16)was transfected by electroporation and clones isolated by limiting dilution in the presence of 1.0 mg/ml of G418. Expression of the PRLR or the EGFR-EPOR on the cell surface was confirmed by flow cytometric analysis using the anti-PRLR (Affinity Bioreagents, MA1-610) or anti-EGFR antibodies (Amersham, RPN.513). For Stat5(Y694F) expressing cells, two clones (A and B) showing different levels of Stat5 expression were further analyzed.

Electrophoretic mobility shift analysis (EMSA). Nuclear extracts were prepared as described previously (17) and EMSA was performed using a prolactin response element (PRE) in the bovine β-casein promoter(15).

Cell lysis and immunoprecipitation. Immunoprecipitation was carried out as described previously(10,17). The proteins were immunoprecipitated from SKT6 cell lysates with either the anti-Jak2 antibody (UBI), anti-Stat5 (raised against the N-terminal region of ovine Stat5)(10), anti-Shc (UBI) or anti-EPOR (kindly provided by Dr. A. Yoshimura). Precipitated proteins were then probed with the monoclonal anti-phosphotyrosine antibody (4G10, UBI) and visualized by the ECL system (Amersham). In some experiments, blots were stripped with stripping buffer and reprobed with the appropriate antibodies. Anti-Stat5 monoclonal antibody was from Transduction Laboratories.

Benzidine staining. Cells treated without or with EPO, PRL, EGF for four days were stained with benzidine. At least three experiments were performed independently.

RESULTS

EPO Stimulates the Tyrosine Phosphorylation of the EPOR, Jak2, and Stat5 in SKT6 Cells

To gain some insight into the EPO-induced pathways involved in erythroid differentiation, we used SKT6 cells. SKT6 cells proliferate without EPO and undergo limited erythroid differentiation in response to EPO (14). Previous studies have shown that EPO induces the tyrosine phosphorylation of various signaling proteins, including the EPOR, Jak2, Stat5, Vav and Shc (17-23) and also stimulates the association of HCP, Syp and phosphatidylinositol 3-kinase (PI3K) with the EPOR (24–28). We sought signaling molecules activated upon EPO stimulation in SKT6 cells. The EPOR, Jak2 and Stat5 were found to be tyrosine phosphorylated in response to EPO (Fig. 1A, B, and C). Stat5 activity was also monitored by electrophoretic mobility shift assays (EMSA) (Fig. 1D). In contrast, EPO failed to activate Tyk2, Jak1, Jak3, Stat1 and Stat3. Furthermore, other signaling molecules such as Shc, PI3K, and Vav were constitutively tyrosine phosphorylated and no additional phosphorylation was detected upon EPO stimulation (Fig. 1E and data not shown). Erythroid differentiation was monitored by benzidine staining. Fifty percent of the SKT6 cells became benzidine positive after 4 days of exposure to EPO, while nine percent of the cells were benzidine positive in the absence of EPO, [(14) see Fig. 2C, 2D, and 6D]. The cell proliferation was neither enhanced nor impaired by the presence of EPO (data not shown). These results show that only Jak2 and Stat5 might be immediately activated upon EPO stimulation.

Prolactin (PRL) Can Substitute for EPO in Signaling Hemoglobin Synthesis

Next we asked whether hemoglobin could be induced by another cytokine receptor shown to activate the Jak2-Stat5 or hemoglobin induction was specific for the EPOR. To test this possibility, the prolactin receptor (PRLR), which plays a role in milk protein expression and activates Jak2 and Stat5 (15,29), was ectopically expressed in SKT6 cells. Stimulation of transfected cells with prolactin (PRL) led to tyrosine phosphorylation of Jak2 and Stat5, though the levels were slightly less than that observed with EPO (Fig. 2A and B). Importantly, PRL was as potent as EPO in stimulating cells to become benzidine positive (Fig. 2C and D). Addition of PRL to the parental cells failed to induce these biochemical events and hemoglobin (data not shown). These data imply that EPO-dependent hemoglobin induction can be achieved by another cytokine receptor PRLR.

EGF Induces hemoglobin Synthesis in STK6 Cells Expressing a Chimeric Receptor Composed of the Extracellular Domain of the EGF Receptor (EGFR) and the Intracellular Domain of the EPOR

Previous studies have shown that chimeric receptors (EGFR-EPOR) carrying the extracellular domain of the human EGF receptor and the intracellular domain of the murine EPO receptor are functional in EPO-responsive TSA-8 cells (30,31). We confirmed this by expressing the EGFR-EPOR in SKT6 cells. Stimulation of the EGFR-EPOR with EGF led to tyrosine phosphorylation of Jak2 and Stat5 in an EGF dose-dependent manner (Fig. 3A, B). EGF at 6 ng/ml induced tyrosine phosphorylation of both Jak2 and Stat5 to a level comparable to that seen with EPO. EGF failed to induce tyrosine phosphorylation of the endogenous EPOR, whereas it stimulated that of the EGFR-EPOR (Fig. 3C). EGF also promoted the expression of hemoglobin

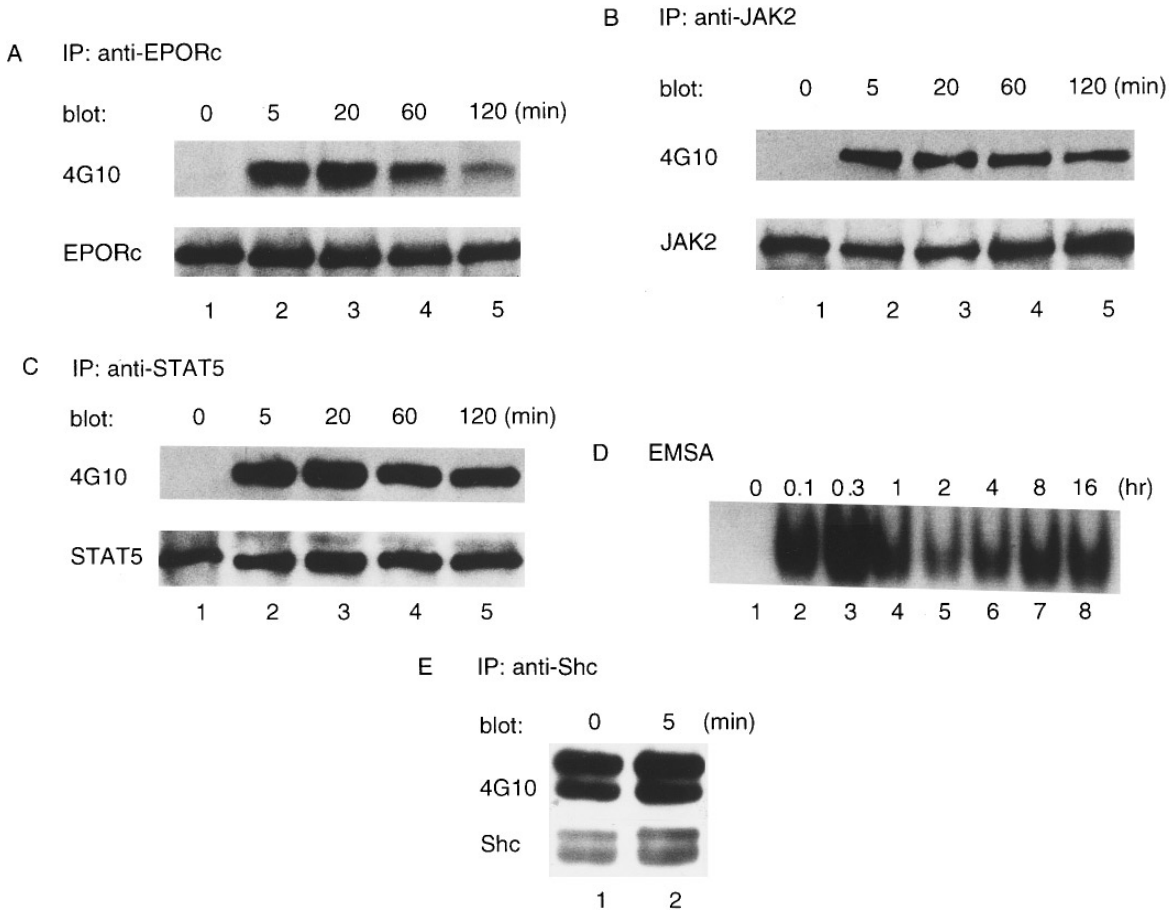

FIG. 1. EPO induces tyrosine phosphorylation of the EPOR, Jak2, and Stat5 in SKT6 cells. (A) EPOR tyrosine phosphorylation in response to EPO. Lysates from SKT6 cells left untreated (lane 1) or stimulated with EPO for the indicated times (lanes 2–5) were subjected to immunoprecipitation with anti-EPOR antibody and blotted with 4G10 (upper panel). Reprobing with anti-EPOR antibody confirmed equal loading (lower panel). (B) Jak2 activation upon EPO stimulation. Cells treated as described above were subjected to immunoprecipitation with anti-Jak2 antibody and blotted with 4G10 (upper panel). Reprobing with anti-Jak2 antibody (lower panel). (C) Tyrosine phosphorylation of Stat5 induced by EPO. Cells treated as described above were subjected to immunoprecipitation with anti-Stat5 antibody and subsequently blotted with 4G10 (upper panel). Reprobing with anti-Stat5 antibody confirmed equal loading (lower panel). (D) EPO-induced Stat5 DNA binding activity. Nuclear extracts from cells treated with EPO for the indicated times or left untreated were subjected to electrophoretic mobility shift assays (EMSA) using ^{32}P-labeled PRE (15). (E) Shc tyrosine phosphorylation with or without EPO challenge. Lysates from cells stimulated with EPO for 5 min or left unstimulated were subjected to immunoprecipitation with anti-Shc antibody and blotted with 4G10 (upper panel). Reprobing with anti-Shc antibody confirmed equal loading (lower panel). The concentration of EPO was 0.5 U/ml throughout this study.

via the EGFR-EPOR while it failed to do so in parental cells (Fig. 3D, data not shown). With regards to Jak2 and Stat5 stimulation, 6 ng/ml of EGF was sufficient to induce hemoglobin synthesis to the same degree as obtained with EPO (Fig. 3D). This amount of EGF stimulated 50 % of the cells to become benzidine positive. These results suggest a correlation between Jak2-Stat5 activation and hemoglobin expression.

Tyrosine Residues within the EPOR are Critical for Hemoglobin Production

We previously showed the importance of tyrosine residues in the mouse EPOR for EPO-dependent proliferation. Both EPO-induced proliferation and Stat5 activation were primarily mediated through tyrosine 343 (10). Since Stat5 activation correlated with EPO- or EGF-driven hemoglobin synthesis, the role of tyrosine residues in the murine EPOR was assessed. To address this issue, mutated chimeric receptors, in which tyrosine 343 was converted to phenylalanine, i.e. EGFR-EPOR(Y343F), or in which either all eight tyrosine residues were substituted with phenylalanines, i.e. EGFR-EPOR(NULL) were expressed in SKT6 cells. EGF stimulation via the EGFR-EPOR(Y343F) and the EGFR-EPOR(NULL) resulted in tyrosine phosphorylation of Jak2 in a dose dependent fashion similar to that observed in cells expressing the EGFR-EPOR (Fig. 3A, 4A and 5A). EGF induced tyrosine phosphorylation of the EGFR-EPOR(Y343F) but not that of the

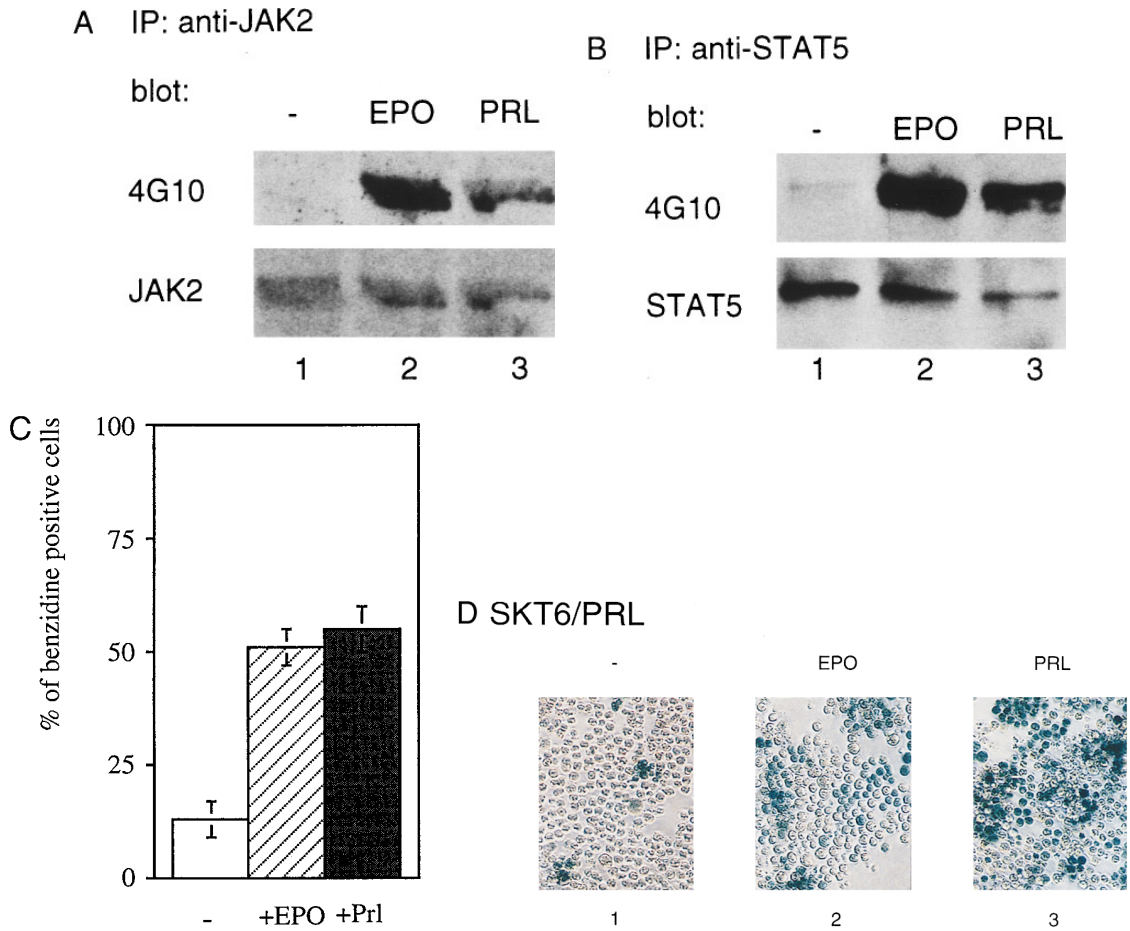

FIG. 2. Prolactin (PRL) induces the tyrosine phosphorylation of Jak2 and Stat5 and the expression of hemoglobin. (A) Activation of Jak2 in response to EPO and PRL. Lysates from PRLR-expressing cells left untreated (lane 1), or stimulated with EPO (lane 2) or PRL (1 μg/ml)(lane 3) for 20 min were subjected to immunoprecipitation with anti-Jak2 antibody and blotted with 4G10 (upper panel). Reprobing with anti-Jak2 antibody confirmed equal loading (lower panel). (B) Stat5 tyrosine phosphorylation by EPO and PRL. Lysates obtained as described above were subjected to immunoprecipitation with anti-Stat5 antibody and blotted with 4G10 (upper panel). Reprobing with anti-Stat5 antibody confirmed equal loading (lower panel). (C) Benzidine positive cell percentage after PRL or EPO treatment. PRLR-expressing cells were left unstimulated or stimulated with EPO or PRL (1 μg/ml) for four days and stained with benzidine. The percentage of benzidine positive cells were shown with the error bars. The data were means of three independent experiments. (−), PRLR-expressing cells without any stimulation. (+EPO), the cells stimulated with EPO. (+PRL), the cells stimulated with PRL. (D) Benzidine staining of PRLR-expressing cells. PRLR expressing cells were stained with benzidine after four days incubation without any stimulation (−), with EPO (+EPO), and with PRL (1 μg/ml) (+PRL). Green-stained cells are benzidine positive.

EGFR-EPOR(NULL) (data not shown). Although tyrosine phosphorylation of Stat5 was normal in response to EPO in both cell lines (Fig. 4B, 5B, lanes 2), it was significantly reduced in EGFR-EPOR(Y343F) expressing cells (Fig. 4B, lanes 3-7), and was completely lost in EGFR-EPOR(NULL) expressing cells upon EGF treatment (Fig. 5B, lanes 3-7). Increase of EGF resulted in an augementation of benzidine positive cell population up to 30 % in EGFR-EPOR(Y343F) expressing cells (Fig 4C). In EGFR-EPOR(NULL) expressing cells, even the highest concentration of EGF tested (600 ng/ml) could not activate Stat5, though the activation of Jak2 could be detected at 0.6 ng/ml (Fig. 5A and B). Less than 10 % of cells became benzidine positive by EGF (Fig. 5C). Thus the ability to activate Stat5 correlated with an increase of the benzidine positive cells (Fig. 4C and 5C).

Dominant Negative Stat5 Inhibits EPO-Induced Erythroid Differentiation

While the data presented above demonstrate a strong correlation between Stat5 activation and hemoglobin synthesis in SKT6 cells, they do not prove that Stat5 is involved in this process. To test this possibility more directly, we overexpressed a dominant negative form of Stat5 in STK6 cells. The tyrosine residue at the position 694 of ovine Stat5 is critical for its DNA binding activity and a substitution of this tyrosine with phenylalanine abolishes the DNA binding ability of

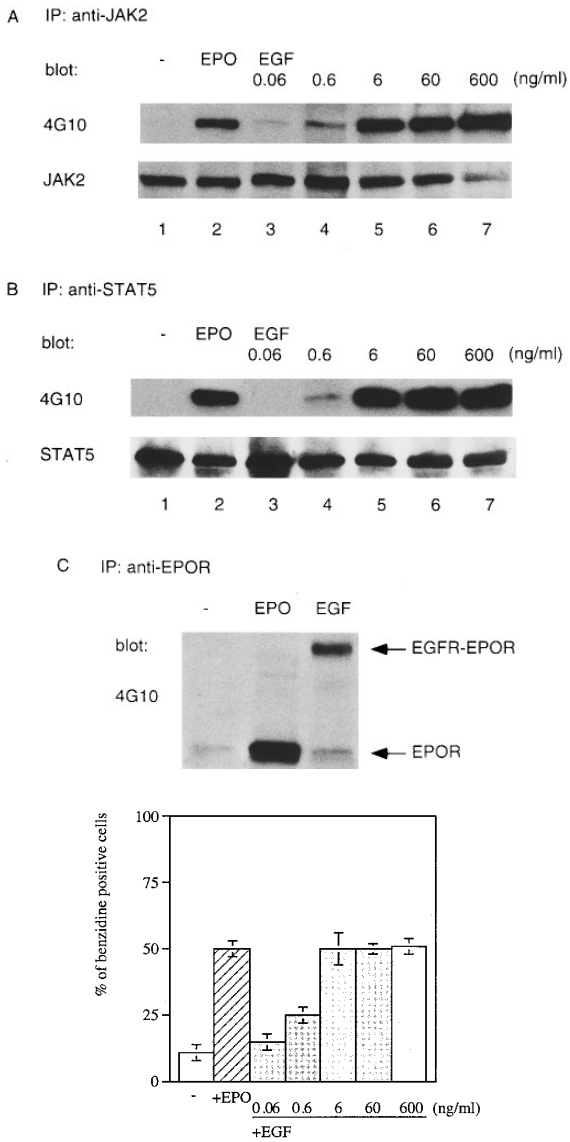

FIG. 3. EGF induces the tyrosine phosphorylation of the EGFR–EPOR chimeric receptor, Jak2, Stat5, and hemoglobin. (A) Activation of Jak2 in response to EPO and EGF. Cells expressing the EGFR–EPOR were left untreated (lane 1), or stimulated with EPO (lane 2) or with the indicated amount of EGF (lanes 3–7) for 20 min. Resulting cell lysates were subjected to immunoprecipitation with anti-Jak2 antibody and blotted with 4G10 (upper panel). Reprobing with anti-Jak2 antibody confirmed equal loading (lower panel). (B) Stat5 tyrosine phosphorylation by EPO and EGF. Cell lysates prepared as described above were subjected to immunoprecipitation with anti-Stat5 antibody and blotted with 4G10 (upper panel). Reprobing with anti-Stat5 antibody confirmed equal loading (lower panel). (C) EGF-dependent EGF–EPOR tyrosine phosphorylation. Lysates from the EGFR–EPOR expressing cells left unstimulated (−), or stimulated with EPO (EPO) or EGF (6 ng/ml) (EGF) for 20 min were subjected to immunoprecipitation with anti-EPOR antibody and blotted with 4G10. (D) Benzidine positive cell percentage after EPO or EGF treatment. Cells expressing the EGFR–EPOR were left unstimulated or stimulated with EPO or with the indicated amount of EGF for four days and stained with benzidine. The data were shown as means of three independent experiments with the error bars. (−), cells without any stimulation. (+EPO), cells stimulated with EPO. (+EGF), cells stimulated with the different amount of EGF (0.06-600 ng/ml).

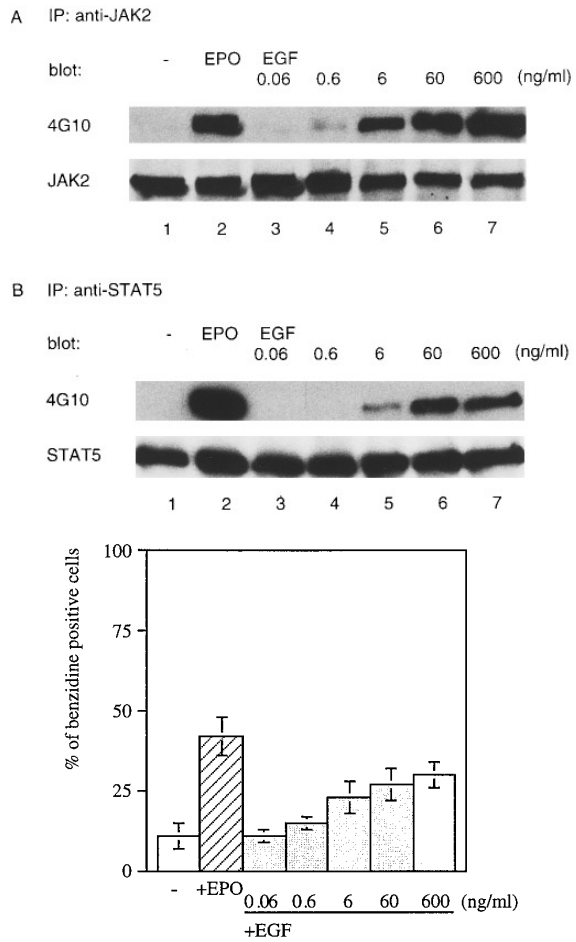

FIG. 4. Activation of Jak2 and Stat5 and induction of hemoglobin in cells expressing the EGFR–EPOR(Y343F). (A) Activation of Jak2 in response to EPO and EGF. (B) Stat5 tyrosine phosphorylation by EPO and EGF. (C) Benzidine positive cell percentage after EPO or EGF treatment. All procedures were same as described in Fig. 3 except that cells expressing the EGFR–EPOR(Y343F) were used in each experiment.

Stat5 (16). Therefore we reasoned that this form of Stat5 could serve as a dominant negative (dn) molecule. This mutated Stat5 cDNA was introduced into SKT6 cells. We analyzed two representative clones that expressed different amounts of dnStat5. SKT6 cells transfected with a dnStat5 cDNA expressed more than five times Stat5 protein present in non-transfected cells as judged by the Stat5 Western blotting(Fig. 6A, lanes 1, 2 and 5 and 6). Stat5 expression was not affected by the presence of EPO (Fig. 6A). The expression of the dnStat5 inhibited tyrosine phosphorylation of Stat5, whereas that of Jak2 was unaffected (Fig. 6B and C). The more dnStat5 that was produced, the less Stat5 that was tyrosinephosphorylated by EPO (Fig. 6A, lanes, 2, 4 and 6, 6C lanes 1, 2 and 3). This impaired tyrosine phosphorylation of Stat5 led to decreased numbers of benzidine-positive cells (i.e. 5-10 %) in re-

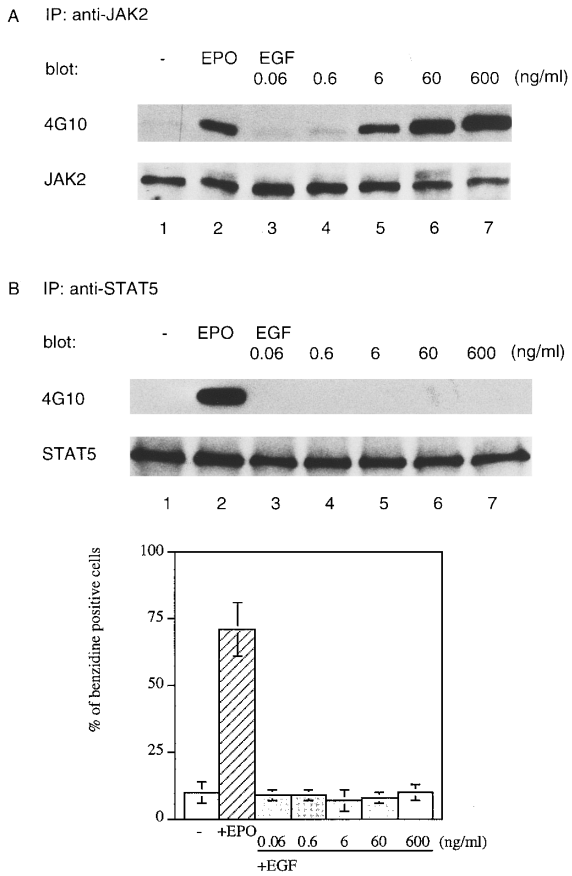

FIG. 5. Activation of Jak2 and Stat5 and induction of hemoglobin in cells expressing the EGFR–EPOR(Null). (A) Activation of Jak2 in response to EPO and EGF. (B) Stat5 tyrosine phosphorylation by EPO and EGF. (C) Benzidine positive cell percentage after EPO or EGF treatment. All procedures were same as described in Fig. 3 except that cells expressing the EGFR–EPOR(Null) were used in each experiment.

sponse to EPO (Fig. 6D). In addition, expression of dnStat5 reduced the basal level of hemoglobin expression (Fig. 6D). We also investigated the effect of dnStat5 overexpression on the other signaling molecules such as Grb-2, Shc, Vav, phosphatidylinositol 3 kinase (PI3K) and c-cbl and found no effect on these proteins (data not shown). These results demonstrate that Stat5 plays a role in EPO-induced hemoglobin synthesis in SKT6 cells.

DISCUSSION

In this study, we used SKT6 cells to understand the role of the EPO-mediated signals in erythroid differentiation (14). This erythroid cell proliferates without EPO and this may be due to the virus envelope glycoprotein gp55, which mimics the action of EPO by binding to the EPOR (32). We found constitutive tyrosine phosphorylation of Shc, Grb-2, Vav, PI3K, c-cbl, SHIP (33) as well as the c-myc expression, whereas phosphorylation of the EPOR, Jak2, and Stat5 was EPO-inducible (Fig. 1A-D, data not shown). These constitutively activated molecules may be responsible for the autonomous cell proliferation. Since EPO eventually induces hemoglobin, our results suggest that Jak2-Stat5 activation may be related to the hemoglobin synthesis, though this pathway is not required for proliferation in this particular cell line. That the ectopic PRLR expression in SKT6 cells led to PRL-dependent tyrosine

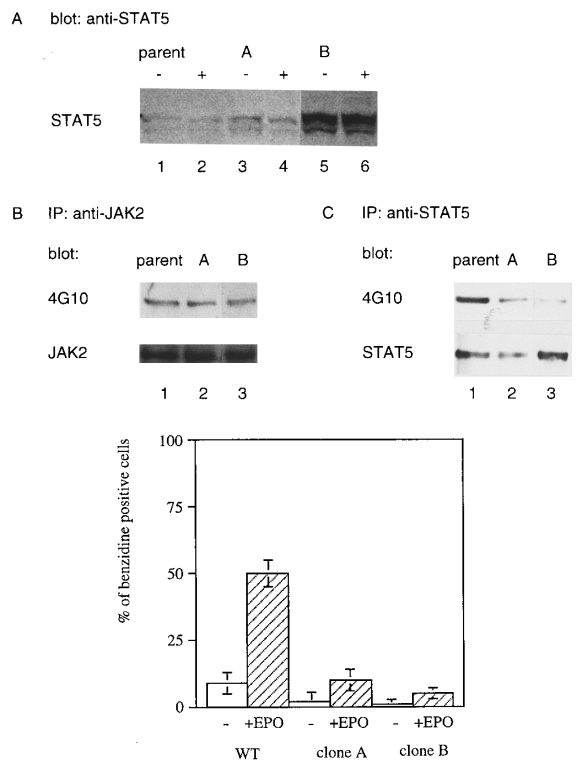

FIG. 6. Expression of dominant negative Stat5 results in impaired tyrosine phosphorylation of Stat5 and decreased hemoglobin production. (A) Stat5 expression in parental cells (lanes 1 and 2) and in cells harboring dominant negative (dn) Stat5 cDNA (lanes 3 and 4, clone A; lanes 5 and 6, clone B). Parental cells and dnStat5 expressing cells were cultured in the absence (−) or in the presence of EPO (+) for 4 days. Cell lysates were separated on SDS-PAGE and blotted with an anti-Stat5 monoclonal antibody. (B) Activation of Jak2 in response to EPO. Cells lysates from parental (lane 1) and from dnStat5 expressing cells (lanes 2 and 3, clones A and B) treated with EPO for 20 min were subjected to immunoprecipitation with anti-Jak2 antibody and blotted with 4G10 (upper panel). The same membrane was reprobed with anti-Jak2 antibody (lower panel). (C) Dominant negative Stat5 compromises Stat5 tyrosine phosphorylation in response to EPO. Cell lysates were prepared as described above and used for immunoprecipitation with anti-Stat5 antibody and blotted with 4G10 (upper panel). Reprobing with anti-Stat5 antibody confirmed equal loading (lower panel). (D) Dominant negative Stat5 expression reduces the EPO-induced benzidine positive cell population. Wild type cells (WT), clone A and clone B were stimulated with (+EPO) or without EPO (−) for four days and stained with benzidine. The percentage of benzidine positive cells were shown with error bars. Data were means of three independent experiments.

phosphorylation of Jak2 and Stat5 (Fig. 2A, B) and resulted in hemoglobin expression (Fig. 2C and D) imply that a signal common between EPOR and PRLR is responsible for the erythroid differentiation. Since a truncated EGFR-EPOR chimeric receptor devoid of the C-terminal half of the cytoplasmic domain responsible for the ras signaling still induced limited erythroid differentiation (31), it is conceivable that Jak2-Stat5 signal emanating from the membrane proximal domain is important for the induction of the differentiation. We confirmed Jak2-Stat5 activation as well as hemoglobin synthesis via EGFR-EPOR chimeric receptor (Fig. 3). We have further evaluated the role of the tyrosine residues in the cytoplasmic domain of the murine EPOR. Although EGF activated Jak2 normally through these mutant receptors, Stat5 activation was severely impaired and hemoglobin expression was decreased (Fig. 4A, B, and C and 5A, B and C). These data indicate that these tyrosines are important for the hemoglobin expression. On the contrary, it has been reported that no tyrosine residue in the EPOR is required for the induction of the β-globin mRNA in Ba/F3 cells which ectopically express the EPOR(6). At present the reason for this discrepancy is unknown. Further study will be necessary to evaluate the role of the tyrosine residues in the EPOR in *in vivo* erythroid cell differentiation.

As Stat5 activation correlated with cytokine-mediated induction of hemoglobin, we overexpressed a mutated Stat5 in STK6 cells to assess directly its role(s) in hemoglobin expression (Fig. 6). We used a mutant Stat5 (Y694 F) which is defective in tyrosine phosphorylation necessary for PRL-, growth hormone- and EPO-induced DNA binding (16,34). Though tyrosine phosphorylation of Stat5 was severely inhibited by expressing dnStat5, it did not affect that of Jak2 (Fig. 6B and C). This inhibition of Stat5 eventually led to a decrease of benzidine positive cells (Fig. 6D). However, decrease of the basal hemoglobin expression was also noticed (Fig. 6D). This may be due to the EPO-independent activation of EPOR-Jak2-Stat5 by gp55, for some autonomous tyrosine phosphorylation of the EPOR in the absence of EPO was detected with longer exposures of the Western blots (data not shown). That dnStat5 overexpression did not interfere with the activation of the other signaling molecules such as Grb-2, Shc, PI3K, Vav and c-cbl (data not shown) suggests that Stat5 sets off the cascades of signals leading to hemoglobin synthesis. Similar results were obtained with another cell line that differentiates in response to EPO (Yoshimura *et al.*, personal communication). In contrast, a correlation between EPO-induced differentiation and the impairement of Stat5 activation has been shown in TF-1 cells(35). These data, however, still reserve the possibility that Stat5 activation is required for EPO-dependent differentiation.

Since the optimal hemoglobin expression was observed after 4 days incubation with EPO, Stat5 may regulate the expression of a key gene(s) which controls globin gene expression. Therefore searching for more EPO-inducible early genes will be of prime importance for the elucidation of erythroid differentiation.

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REFERENCES

1. D'Andrea, A. D. (1994) *Curr. Opin. Cell Biol.* **6,** 804–808.
2. Liboi, E., Carroll, M., D'Andrea, A., and Mathey-Prevot, B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11,351–11,355.
3. Chiba, T., Nagata, Y., Kishi, A., Sakamaki, K., Miyajima, A., Yamamoto, M., Engel, J. D., and Todokoro, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11,593–11,597.
4. Carroll, M., Zhu, Y., and D'Andrea, A. D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 14,964–14,969.
5. Krosl, J., Damen, J. E., Krystal, G., and Humphries, R. K. (1995) *Blood* **85,** 50–56.
6. Krosl, J., Damen, J. E., Krystal, G., and Humphries, R. K. (1996) *J. Biol. Chem.* **271,** 27,432–27,437.
7. Nishijima, I., Nakahata, T., Hirabayashi, Y., Inoue, T., Kurata, H., Miyajima, A., Hayashi, N., Iwakura, Y., Arai, K., and Yokota, T. (1995) *Mol. Biol. Cell.* **6,** 497–508.
8. Ihle, J. N. (1995) *Nature* **377,** 591–594.
9. Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T., and Miyajima, A. (1995) *EMBO J.* **14,** 2816–2826.
10. Damen, J. E., Wakao, H., Miyajima, A., Krosl, J., Humphries, R. K., Cutler, R. L., and Krystal, G. (1995) *EMBO J.* **14,** 5557–5568.
11. Friedmann, M., Migone, T., Russell, S., and Leonard, W. (1995) *Proc. Natl. Acad. Sci. USA* **93,** 2077–2082.
12. Gobert, S., Chretien, S., Gouilleux, F., Muller, O., Pallard, C., Dusanter-Fourt, I., Groner, B., Lacombe, C., Gisselbrecht, S., and Mayeux, P. (1996) *EMBO J.* **15,** 2434–2441.
13. Mui, A. L.-F., Wakao, H., Kinoshita, T., Kitamura, T., and Miyajima, A. (1996) *EMBO J.* **15,** 2425–2433.
14. Todokoro, K., Kanazawa, S., Amanuma, H., and Ikawa, Y. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4126–4130.
15. Wakao, H., Gouilleux, F., and Groner, B. (1994) *EMBO J.* **13,** 2182–2191.
16. Gouilleux, F., Wakao, H., Mund, M., and Groner, B. (1994) *EMBO J.* **13,** 4361–4369.
17. Wakao, H., Harada, N., Kitamura, T., Mui, A. L., and Miyajima, A. (1995) *EMBO J.* **14,** 2527–2535.
18. Carroll, M. P., Spivak, J. L., MacMahon, M., Weich, N., Rapp, U. R., and May, W. S. (1991) *J. Biol. Chem.* **266,** 14,964–14,969.
19. Miura, O., D'Andrea, A. D., Kabat, D., and Ihle, J. N. (1991) *Mol. Cell. Biol.* **11,** 4895–4902.
20. Quelle, F. W., and Wojchowski, D. M. (1991) *J. Biol. Chem.* **266,** 609–614.

21. Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J. N. (1993) *Cell* **74,** 227–236.
22. Miura, O., Miura, Y., Nakamura, N., Quelle, F. W., Witthuhn, B. A., Ihle, J. N., and Aoki, N. (1994) *Blood* **84,** 4135–4141.
23. Damen, J. E., Liu, L., Culter, R. L., and Krystal, G. (1993) *Blood* **82,** 2296–2303.
24. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) *Cell* **80,** 729–738.
25. Yi, T., Zhang, J., Miura, O., and Ihle, J. N. (1995) *Blood* **85,** 87–95.
26. Tauchi, T., Feng, G. S., Shen, R., Hoatlin, M., Bagby, G. C. J., Kabat, D., Lu, L., and Broxmeyer, H. E. (1995) *J. Biol. Chem.* **270,** 5631–5635.
27. Damen, J. E., Mui, A. F., Puil, L., Pawson, T., and Krystal, G. (1993) *Blood* **81,** 3204–3210.
28. Miura, O., Nakamura, N., Quelle, F. W., Witthuhn, B. A., Ihle, J. N., and Aoki, N. (1994) *J. Biol. Chem.* **269,** 614–620.
29. Rui, H., Lebrun, J. J., Kirken, R. A., Kelly, P. A., and Farrar, W. L. (1994) *Endocrinology* **135,** 1299–1306.
30. Ohashi, H., Maruyama, K., Liu, Y.-C. Y., and Yoshimura, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 158–162.
31. Maruyama, K., Miyata, K., and Yoshimura, A. (1994) *J. Biol. Chem.* **269,** 5976–5980.
32. Yoshimura, A., Longmore, G., and Lodish, H. (1990) *Nature* **348,** 647–649.
33. Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., and Krystal, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1689–1693.
34. Gouilleux, F., Pallard, C., Dusanter-Fourt, I., Wakao, H., Haldosen, L. A., Norstedt, G., Levy, D., and Groner, B. (1995) *EMBO J.* **14,** 2005–2013.
35. Chretien, S., Varlet, P., Verdier, F., Gobert, S., Cartron, J.-P., Gisselbrecht, S., Mayeux, P., and Lacombe, C. (1996) *EMBO. J.* **15,** 4174–4181.