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Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5 activation

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FDCP-1 cells are hematopoietic progenitor cells which require interleukin-3 for survival and proliferation. FDCP-1 cells stably transfected with the murine erythropoietin receptor cDNA survive and proliferate in the presence of erythropoietin. Erythropoietin induces the activation of the short forms (80 kDa) of STAT5 in the cells. Erythropoietin-induced activation of STAT5 was strongly reduced in cells expressing mutated variants of the erythropoietin receptors in which tyrosine residues in their intracellular domain have been eliminated. We determined that the erythropoietin receptor tyrosine residues 343 and 401 are independently necessary for STAT5 activation. The amino acid sequences surrounding these two tyrosine residues are very similar. Peptides comprising either phosphorylated Tyr343 or phosphorylated Tyr401, but not their unphosphorylated counterparts, inhibited the STAT5 activation. We propose that these two tyrosine residues of the erythropoietin receptor constitute docking sites for the STAT5 SH2 domain. The growth stimulus mediated by erythropoietin was decreased in cells expressing erythropoietin receptors lacking both Tyr343 and Tyr401. This suggests that STAT5 activation could be involved in the growth control of FDCP-1 cells.

Keywords: erythropoietin/hematopoiesis/STATs/tyrosine phosphorylation

Introduction

Erythropoietin (Epo) is essential for the survival and proliferation of the late erythroid progenitors and their differentiation into erythrocytes (Koury and Bondurant, 1992). The Epo-receptor (Epo-R) belongs to the cytokine receptor superfamily (D'Andrea *et al.*, 1989; Bazan, 1990). Ligand stimulation of these receptors induces the tyrosine phosphorylation of many proteins including the receptors themselves. The activation of tyrosine kinases of the JAK family (JAK1–3 and TYK2) constitutively associated with a membrane-proximal domain of the intracellular part of the cytokine receptors, initiates the intracellular transduction signalling process (Stahl and Yancopoulos, 1993). Other tyrosine kinases, including members of the src family, also appear to be activated (see Taniguchi, 1995 for review). It has not been determined definitively whether JAK kinases are responsible for the tyrosine phosphorylation of the receptors.

Among the JAK kinase substrates are the transcription factors of the STAT family (Ihle and Kerr, 1995). The STATs (Signal Transducers and Activators of Transcription) are transcription factors activated by tyrosine phosphorylation in the cytoplasm or in the vicinity of the cytoplasmic membrane before translocation to the nucleus. The STAT proteins possess SH2 and SH3 domains. It has been proposed that STAT tyrosine phosphorylation enables their homo- or hetero-dimerization by association of the phosphorylated tyrosine of one STAT molecule with the SH2 domain of another STAT molecule. To date, seven members of the STAT family have been cloned. STAT1 (p91) is activated by interferon (IFNγ) (Shuai *et al.*, 1992), STAT2 (p113) which, together with STAT1 and p48 constitute the transcription factor ISGF-3, is activated by IFN α/β (Fu, 1992; Schindler *et al.*, 1992a,b) and STAT3 (APRF) is activated in response to interleukin (IL)-6 (Akira *et al.*, 1994; Zhong *et al.*, 1994) and granulocyte-colony stimulating factor (G-CSF; Tian *et al.*, 1994). It has recently been shown that STAT4 was phosphorylated in response to IL-12 (Quelle *et al.*, 1995). STAT5 (mammary gland factor, MGF) was initially reported to be activated in response to prolactin. It has been found that growth hormone and hematopoietic cytokines such as Epo, thrombopoietin, IL-2, IL-3 and granulocyte–macrophage colony stimulating factor (GM-CSF) are also able to activate STAT5 (MGF) (Wakao *et al.*, 1994; Azam *et al.* 1995; Gouilleux *et al.*, 1995a,b; Mui *et al.*, 1995; Pallard *et al.*, 1995a,b). The products of two genes encoding MGF-homologues (STAT5A and STAT5B) have been cloned from a murine cell line cDNA (Mui *et al.*, 1995; Azam *et al.*, 1995). STAT6 is activated by IL-4 and by IL-3 (Hou *et al.*, 1994; Quelle *et al.*, 1995).

JAK kinases are able to phosphorylate STAT proteins directly (Yamamoto *et al.*, 1994; Gouilleux *et al.*, 1995a). However, although JAK recruitment by each cytokine appears to exhibit some specificity, the activation of specific STATs by a cytokine cannot only be explained by the type of JAK kinase activated. For example, both Epo and IFNγ activate JAK2 in UT-7 cells, but only Epo activates STAT5 whereas IFNγ activates STAT1 (Pallard *et al.*, 1995a). IL-2, which appears to signal through JAK1 and JAK3, also activates STAT5 (Wakao *et al.*, 1995) while activation of the IL-4 receptor recruits JAK1 and JAK3 but leads to STAT6 activation (Hou *et al.*, 1994; Quelle *et al.*, 1995). Thus, the specificity of STAT activa-

tion appears to be more related to the cytokine receptors themselves than to the activated Jak kinase. An attractive and partly demonstrated model would be that the STAT SH2 domains interact with the tyrosine phosphorylated residues of activated cytokine receptors (Greenlund *et al.*, 1994). This interaction would place the STAT proteins in close vicinity to the receptor-associated JAK kinases which phosphorylate them. In agreement with this hypothesis, recent reports have shown that selection of a STAT by a receptor depends on the presence of particular SH2 domains in the STAT protein (Heim *et al.*, 1995) and on the presence of particular tyrosine-comprising motifs in the receptor (Stahl *et al.*, 1995).

Ligand activation of Epo-R activates the receptor-associated tyrosine kinase JAK2 (Witthuhn *et al.*, 1993). Like most other cytokine receptors, Epo-R becomes tyrosine-phosphorylated after ligand stimulation (Miura *et al.*, 1991; Dusanter-Fourt *et al.*, 1992; Yoshimura and Lodish, 1992). In this paper, we show that Epo stimulation of FDCP-1 cells transfected with the Epo-R activated STAT5 and that two tyrosine residues of the intracellular domain of the Epo-R were required for efficient STAT5 activation. Each of these tyrosine residues was able to independently stimulate STAT5 DNA binding activity to an extent similar to the wild-type receptor. The amino-acid sequences surrounding these two tyrosine residues are closely related and tyrosine-phosphorylated peptides comprising these sequences abbrogated the DNA binding of Epo-activated STAT5 proteins.

Results

Epo activates STAT5 in Epo-R transfected FDCP-1 cells

Transfection of wild-type Epo-R into IL-3-dependent FDCP-1 cells enables these cells to proliferate under Epo stimulation (Quelle and Wojchowsky, 1991). We investigated the induction of STAT5 DNA binding activity in parental FDCP-1 cells and Epo-R transfected FDCP-1 cells. Nuclear extracts were prepared from FDCP-1 cells starved of growth factors for 4 h, stimulated with IL-3 or Epo and tested for the binding of STAT5 to a labelled probe from the β-casein promoter in bandshift experiments (Wakao *et al.*, 1994). IL-3, but not Epo, induced the electrophoretic shift of the radiolabelled probe in parental FDCP-1 cells. Epo and IL-3 induced a DNA–protein complex in Epo-R transfected FDCP-1 cells (Figure 1A). The identity of the DNA–protein complex induced by Epo was investigated. Nuclear extracts from Epo-stimulated cells were incubated with antisera specific for each STAT protein before analysis by EMSA. As shown in Figure 1B, STAT5 specific antibodies, but not STAT1 or STAT3 specific antibodies, supershifted the DNA–protein complex induced by Epo, indicating that a STAT5 or a STAT5-related factor was activated by Epo. Antibodies specific for STAT4 and STAT6 did not supershift the β-casein or IRF-1 probes shifted by nuclear extracts from Epo-stimulated FDCP-1 cells (data not shown). Moreover, a I_ϵ probe (5′-GTCAACTTCCCAAGAACAGAA) was not shifted by these nuclear extracts (data not shown).

Epo-induced DNA binding activity exhibited a specificity similar to that of STAT5-MGF, since an efficient competition of binding was observed with an unlabelled

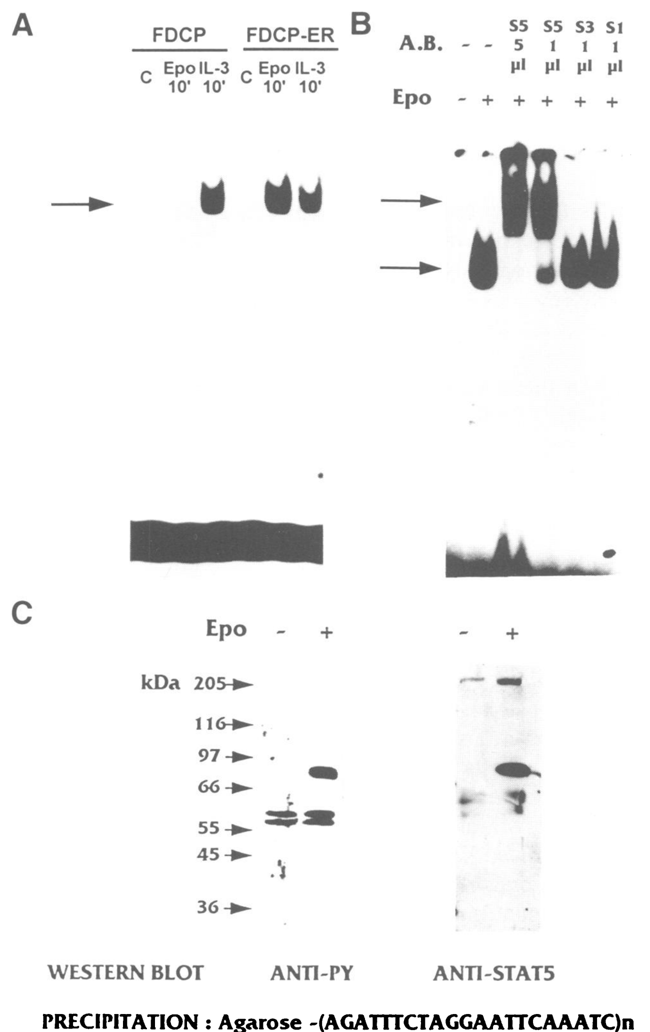

Fig. 1. Epo activates STAT5 in Epo-R-transfected FDCP-1 cells. (**A**) Growth factor-deprived untransfected FDCP-1 cells (FDCP) or FDCP-1 cells transfected with the wild-type Epo-R (FDCP-ER) were stimulated for 10 min with either 10 U/ml Epo or 10 ng/ml IL-3. Nuclear extracts were then prepared and tested for EMSA using a β-casein probe. (**B**) FDCP-ER cells were stimulated or not with 10 U/ml Epo for 10 min and nuclear extracts were prepared. Nuclear extracts were incubated with antibodies against STAT5, STAT3 or STAT1 and analysed by EMSA. (**C**) Nuclear extracts from 120×10^6 Epo-stimulated or control cells were incubated with multimerized β-casein oligonucleotides covalently bound to agarose. After extensive washing, bound material was eluted by boiling into Laemmli sample buffer, separated through a 7.5% polyacrylamide gel and analysed by Western blot using successively anti-phosphotyrosine (anti-PY) and anti-STAT5 antibodies.

IRF-1, GAS oligonucleotide, but not with a SIE oligonucleotide (data not shown). To directly identify the Epo-induced DNA binding activity, nuclear extracts were reacted with multimerized STAT5-MGF binding sites covalently bound to agarose beads (Wakao *et al.*, 1994). The bound proteins were eluted and analysed by Western blot analysis using anti-phosphotyrosine or anti-STAT5 antibodies (Figure 1C). Both antibodies recognized proteins specifically purified from Epo-stimulated cells but not from control cells. These proteins exhibited molecular masses of ~80 kDa. Careful examination of anti-phosphotyrosine Western blots revealed the presence

of two poorly separated proteins with slightly different migration properties. The Epo-induced DNA binding activity in FDCP-1 cells most likely corresponds to the short forms of STAT5A and STAT5B previously identified in IL-3 stimulated immature myeloid cells.

Epo-Rs lacking tyrosine residues in their intracellular domain poorly activate STAT5

We have previously shown that the removal of all the tyrosine residues of the intracellular domain of the Epo-R decreases its ability to transduce a mitogenic signal in IL-3 dependent murine hematopoietic cells. We tested the ability of these receptors to mediate Epo-induced STAT5 activation. As shown in Figure 2A, Epo-induced β-casein probe binding was strongly impaired in FDCP-1 cells expressing Epo-Rs without tyrosine residues (ZERO). STAT5 activation by IL-3 was not affected in these cells. The low β-casein probe binding ability induced by Epo was fully supershifted by anti-STAT5 antibodies (data not shown). Kinetic studies showed that Epo-induced STAT5 activation was strongly reduced throughout the tested period (Figure 2B) demonstrating that STAT5 activation was not simply delayed in cells expressing the ZERO mutant. Dose–response curves showed that higher Epo-concentrations were necessary to observe STAT5 activation in cells expressing the ZERO Epo-R mutant (Figure 2C). In order to quantify more accurately the activation level of STAT5 in FDCP-1 cells transfected with the mutated Epo-Rs, EMSA gels were scanned using a PhosphorImager. The results were standardized relative to IL-3 stimulation. In cells transfected with the wild-type receptor, Epo appeared to be nearly as efficient as IL-3 in the activation of STAT5 (Epo/IL-3 = 99 ± 13%; $N = 4$) whereas in ZERO transfected cells, Epo efficiency was strongly reduced (Epo/IL-3 = 8.8 ± 4%; $N = 8$). The same results were obtained in BaF3 cells transfected with the ZERO Epo-R mutant (data not shown).

The numbers of available Epo binding sites at the cell surface of ZERO or wild-type Epo-R-transfected FDCP-1 cells were measured by [^{125}I]Epo binding. FDCP-1 cells transfected with the ZERO Epo-R mutant and selected for growth in Epo-containing medium expressed ~5700 Epo-Rs per cell. Cells transfected with the wild-type Epo-R and selected likewise expressed 2100 Epo-Rs per cell (Table I). Thus, the strongly decreased STAT5 activation in FDCP-1 cells transfected with the ZERO mutant did not result from a reduced Epo-R expression at the cell surface.

Epo-R tyrosines 343 and 401 are involved in STAT5 activation

To determine which Epo-R tyrosines are involved in STAT5 activation, FDCP-1 cells were transfected with substitution and deletion mutants of the Epo-R (Figure 3). Epo activated STAT5 with the same efficiency both in FDCP-1 cells transfected with the Y1 mutant, which only retains a single tyrosine at position 343, and in FDCP-1 transfected with the wild-type receptor (Figure 4). Only a single amino acid at position 343 distinguishes the ZERO and Y1 Epo-R mutants (Figure 3). A tyrosine residue at 343 is sufficient to restore a full STAT5 activation. To determine whether Tyr343 was necessary for Epo-induced STAT5 activation in the context of the full-length receptor,

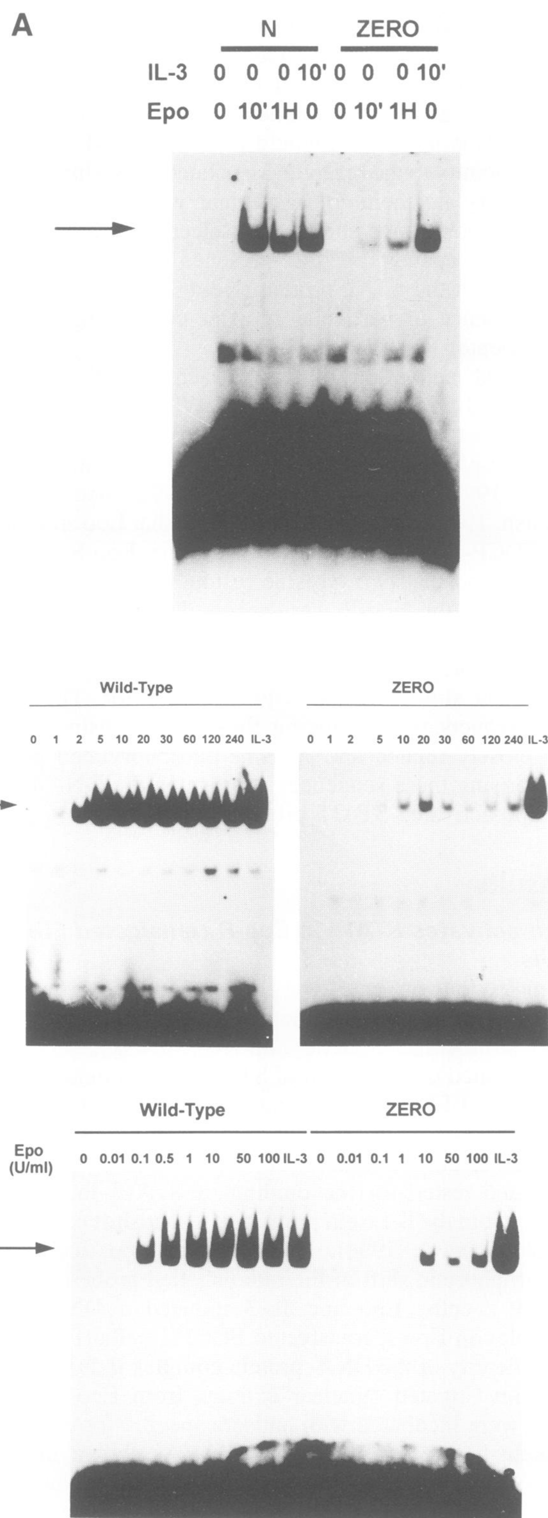

Fig. 2. STAT5 activation mediated by wild-type and ZERO mutant Epo-R. FDCP-1 cells were stably transfected either with wild-type Epo-R or with Epo-R without tyrosine residues in its intracellular domain (ZERO) and selected for ability to grow under Epo stimulation. (**A**) The cells were factor deprived and stimulated with 10 U/ml Epo for 10 or 60 min. (**B**) Kinetic study; the cells were stimulated with 10 U/ml Epo for the indicated times. (**C**) Epo dose–response assay; the cells were stimulated for 10 min with the indicated Epo concentrations. As control, cells were also stimulated with 10 ng/ml IL-3 for 10 min. Nuclear extracts were analysed by EMSA with a β-casein probe.

Table I. Epo binding sites in FDCP-1 cells transfected with Epo-R mutants

Transfected Epo-R	B_{max} (sites per cell; mean + SD, N = 3)
Wild-type	2110 ± 50
F1-Y2-8	1530 ± 40
Y1	2660 ± 95
F1 (ZERO)	5700 ± 113
F1-Y2	9500 ± 290
F1-Y2-3	4050 ± 460
F1-Y2-4	6590 ± 560
F1-Y3-4	7700 ± 210
F1-Y5-8	10340 ± 410

Epo-Rs were measured using a saturating concentration (2 nM) of [^{125}I]Epo. Non-specific binding was determined using a 100-fold molar excess of unlabelled Epo and the results were standardized to a reference cell line (UT-7).

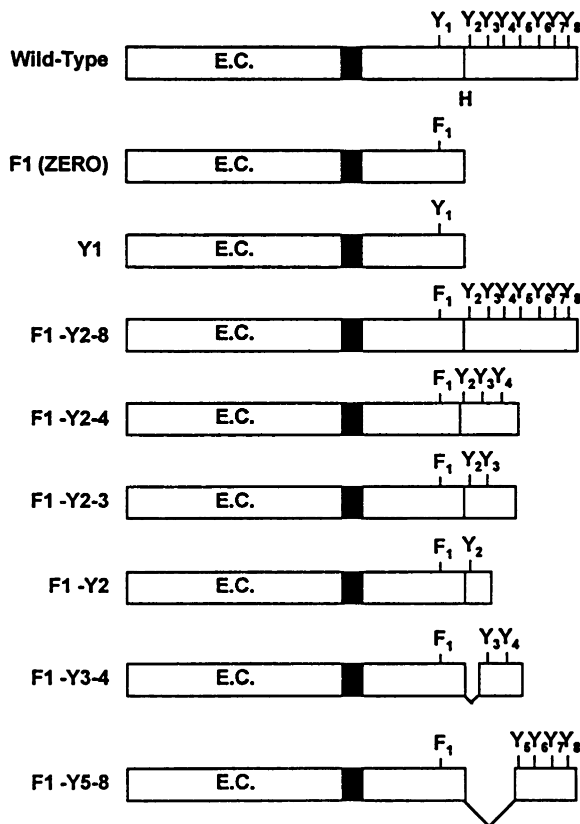

Fig. 3. Schematic representation of normal and mutated Epo-Rs. The first 24 amino acids which constitute the hydrophobic leader sequence are not presented and are not taken into account in numbering. The intracellular part of the wild-type Epo-R contains eight tyrosine residues [positions 343 (Y1), 401 (Y2), 429 (Y3), 431 (Y4), 443 (Y5), 460 (Y6), 464 (Y7) and 479 (Y8)]. The unique *Hin*dIII restriction site is located on the cDNA at a position corresponding to the amino acid 374.

we transfected FDCP-1 cells with a F1-Y2-8 receptor mutant. As shown in Figure 5, Epo strongly activated STAT5 in these cells. This suggests that the function exerted by Tyr343 in Y1 could be redundant in the full-length receptor. To identify additional tyrosines which might confer STAT5 activation, FDCP-1 cells were transfected with Epo-R mutants in which Tyr343 (Y1) was mutated to Phe (F1) and additional mutations were introduced (Figure 3). Only the Epo-R mutants which retained

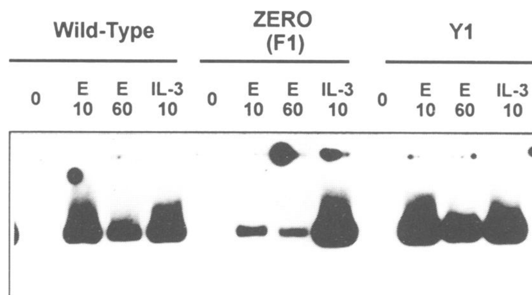

Fig. 4. Tyrosine 343 of the Epo-R allowed full Epo-induced STAT5 activation. FDCP-1 cells were transfected with wild-type, F1 or Y1 Epo-Rs and selected for growth. Starved cells were stimulated with 10 U/ml Epo or with 10 ng/ml IL-3, nuclear extracts were prepared and tested for EMSA using a β-casein DNA probe.

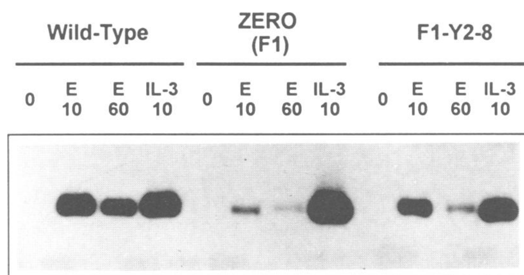

Fig. 5. Mutation of Y^{343} in the wild-type Epo-R did not abbrogate STAT5 activation. FDCP-1 cells were transfected with wild-type, F1 or F1-Y2-8 Epo-Rs and selected for growth. Starved cells were stimulated with 10 U/ml Epo or with 10 ng/ml IL-3, nuclear extracts were prepared and tested for EMSA using a β-casein DNA probe.

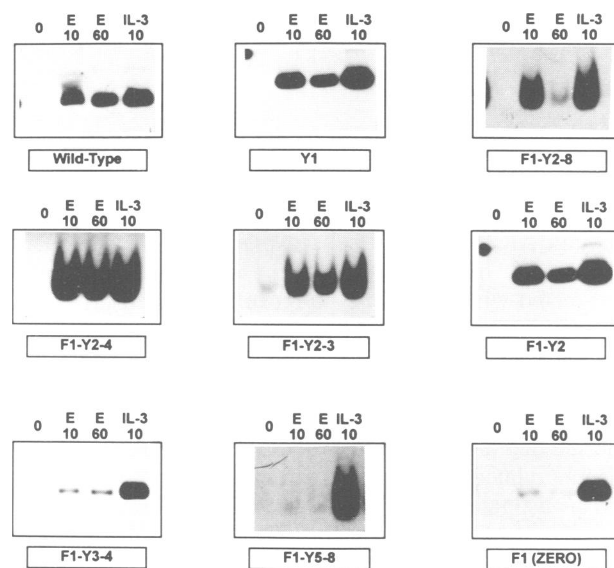

Fig. 6. Epo-stimulated STAT5 activation in FDCP-1 cells expressing various Epo-R mutants. FDCP-1 cells were transfected with the indicated Epo-Rs and selected for growth. Starved cells were stimulated with 10 U/ml Epo or with 10 ng/ml IL-3; nuclear extracts were prepared and tested for EMSA using a β-casein DNA probe.

Tyr401 (namely F1-Y2, F1-Y2-3, F1-Y2-4 and F1-Y2-8 but not F1-Y3-4 or F1-Y5-8) were able efficiently to activate STAT5 in response to Epo (Figure 6). However, mutants with Tyr429 and Tyr431 (F1-Y3-4) reproducibly exhibited an Epo-induced STAT5 activation slightly higher than the ZERO (F1) or the F1-Y5-8 mutants. The Epo-Rs

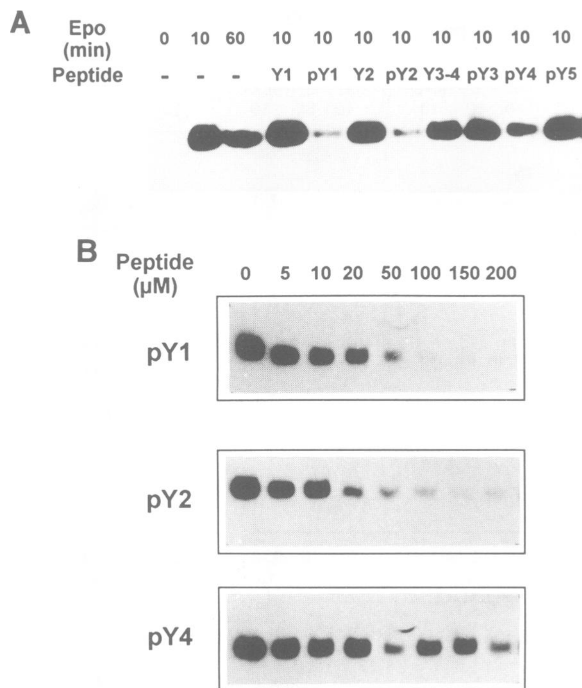

Fig. 7. Inhibition of β-casein DNA probe binding by tyrosine-phosphorylated peptides. FDCP-1 cells expressing wild-type Epo-Rs were stimulated or not with 10 U/ml Epo for 10 or 60 min and nuclear extracts were prepared. (**A**) Nuclear extracts from cells stimulated for 10 min with Epo were incubated for 30 min at 4°C without peptide (–) or with 200 µM of the following peptides: Y1, KAQTYLVLD; pY1, KAQTYpLVLD; Y2, KSSFEYTILD; pY2, KSSFEYpTILD; Y3-4, KHLKYLYLVV; pY3, KSSFEYpLYLVV; pY4, KSSFEYLYpLVV; pY5, KISTDYpSSGG and tested for EMSA using a β-casein DNA probe. (**B**) The same nuclear extracts were incubated with peptide concentrations of pY1, pY2 or pY4 ranging from 5 to 200 µM and processed as above.

expressed at the cell surface were measured by [^{125}I]Epo binding. All the utilized cells expressed Epo-R numbers similar to or higher than that expressed by FDCP-1 cells transfected with the wild-type receptor (Table I).

Tyrosine-phosphorylated peptides comprising Tyr343 or Tyr401 inhibit STAT5 DNA binding activity

The interaction between the STAT5 SH2 domain and the phosphorylated Tyr343 and Tyr401 residues of the Epo-R was investigated. We synthesized several peptides either phosphorylated or not and tested their ability to inhibit DNA binding activity of Epo-activated STAT protein. The peptides were first tested at a high concentration (200 µM) and only phosphorylated peptides corresponding to Tyr343 (Y1), Tyr401 (Y2) and, to a much lesser extent, to Tyr431 (Y4) appreciably inhibited STAT5 DNA binding (Figure 7A). Decreasing concentrations of these peptides were then tested to determine the relative efficiency of each peptide (Figure 7B). Almost complete inhibition of complex formation was obtained with concentrations of 50 µM of phosphopeptide Tyr343 (pY1) or 20 µM of phosphopeptide Tyr401 (pY2). A concentration of 200 µM of phosphopeptide Tyr431 (pY4) was necessary to observe a partial binding inhibition.

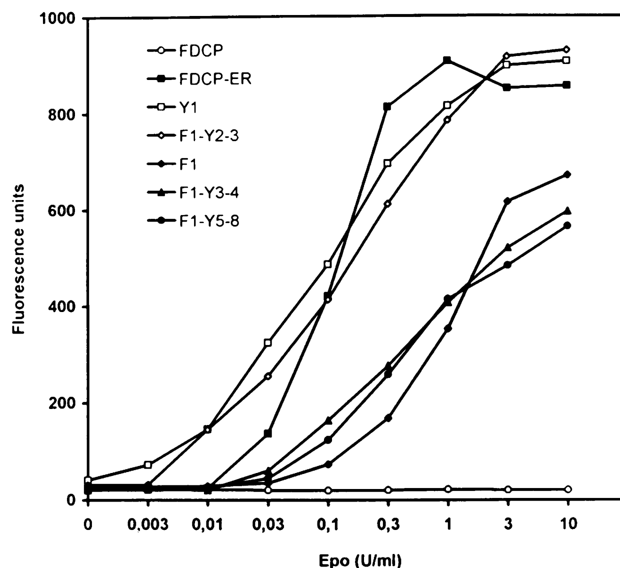

Fig. 8. Epo-stimulated proliferation of FDCP-1 cells transfected with various Epo-R mutants. Non-transfected FDCP-1 cells (FDCP) or cells transfected with the wild-type Epo-R (FDCP-ER) or with various Epo-R mutants were seeded in 96-well microplates with Epo concentrations ranging from 0 to 10 U/ml and incubated for 72 h. Cell proliferation was then determined using AlamarBlue. In the conditions used, fluorescence intensity was linearly proportional to the live cell number.

Epo-induced cell proliferation was decreased in cells expressing Epo-R mutants with low STAT5 activation ability

Epo-induced cell proliferation was measured using a fluorescent analogue of MTT. Figure 8 shows that cells expressing Epo-R mutants with impaired STAT5 activation ability (namely F1, F1-Y3-4 and F1-Y5-8) exhibited a decreased Epo sensitivity. Indeed, the growth of these cells required higher Epo concentrations and thus the stimulation of a higher number of Epo-Rs. Moreover, even at high Epo concentrations, the proliferation of these cells was lower than the proliferation of cells expressing wild-type Epo-Rs, despite the higher Epo-R numbers expressed at their cell surface (Table I).

Discussion

Epo activates STAT5 in Epo-R-transfected FDCP-1 cells

During the last few years, many studies have established that the JAK–STAT pathway is shared by all cytokine receptors (Ihle and Kerr, 1995). However, the specificity of STAT activation is not well understood. STAT5 activation by Epo was previously reported for the Epo-responsive UT-7 cell line and for Epo-R transfected BaF3 cells (Gouilleux *et al.*, 1995b; Pallard *et al.*, 1995a,b). Epo-stimulated activation of STAT1 and STAT3 in the Epo-dependent erythroleukemia cell line HCD-57 was also reported (Ohashi *et al.*, 1995) although Epo-R does not possess the YXXQ motif shown to be necessary for STAT3 activation by the IL-6 and LIF receptors (Stahl *et al.*, 1995). Lastly, in TF-1 cells, Finbloom *et al.* (1994) have shown that Epo stimulated an unidentified STAT protein which was not recognized by anti-STAT1 antibodies. The Epo-activated protein in TF-1 cells was

recently identified as STAT5 (Wakao *et al.*, 1995). In this study we show that Epo activates STAT5 in FDCP-1 cells transfected with the wild-type Epo-receptor. No activation of either STAT1, STAT3, STAT4 or STAT6 was detected in Epo-stimulated FDCP-1 cells as previously reported for UT-7 or BaF3 cells (Pallard *et al.*, 1995a,b). Moreover, Epo stimulation of fetal rat liver CFU-E also activates STAT5 (C.Lacombe, S.Chretien, P.Varlet, S.Gobert, E.Devemy, C.Billat, S.Gisselbrecht and P.Mayeux, manuscript in preparation). Thus, the main Epo-activated STAT appears to be STAT5, although other STATs such as STAT1 and STAT3 could be activated by Epo in some cells.

Our data show that Epo activated the short forms (80 kDa) of STAT5 in FDCP-1 cells, in contrast to UT-7 cells or to Epo-R transfected BaF3 cells where Epo stimulates the long forms (97 kDa) of STAT5 (Pallard *et al.*, 1995a,b). Thus, Epo appears to be able to stimulate both long and short STAT5 forms, depending on the cell lines. The biological significance of this difference is under study. STAT5 activation by Epo was very efficient and overexposed EMSA gels easily allowed the detection of activated STAT5 in cells stimulated with as few as 10 mU/ml Epo. At this Epo concentration, <10 Epo-Rs per cell were occupied by the hormone.

Mechanisms of STAT5 activation by Epo

STAT5 activation by Epo was strongly reduced in cells expressing receptors without tyrosine residues (Figure 2). This low level of activation was not due to a reduced Epo-R expression at the cell surface since these cells expressed more Epo-Rs than cells transfected with the wild-type receptor (Table I). Moreover, Epo-stimulated JAK2 activation was not decreased in cells expressing the F1 Epo-R mutant compared with cells expressing wild-type Epo-Rs (Gobert *et al.*, 1995 and unpublished data). In cells expressing F1 Epo-Rs, STAT5 activation was not detected in cells stimulated with <1 U/ml Epo, indicating that STAT5 activation required the ligand stimulation of >800 Epo-Rs per cell. Whether the low level of STAT5 activation observed in cells expressing receptors without tyrosine is mediated through the direct association of STAT5 with JAK2, as suggested by the ability of JAK2 to phosphorylate STAT5 in a cell-free assay (Gouilleux *et al.*, 1994), or involves adaptator protein(s), remains to be determined.

Our results show that Tyr343 (Y1) and Tyr401 (Y2) of the Epo-R are involved in STAT5 activation. Either of these tyrosines was sufficient to obtain full STAT5 activation (Figures 4, 5 and 6). Phosphopeptides comprising these tyrosine residues inhibited DNA complex formation of STAT5 (Figure 7). Moreover, Epo-R Tyr431 (Y4) could also mediate STAT5 activation but to a much lower extent (Figure 6). Again, a corresponding tyrosine-phosphorylated peptide inhibited binding of activated STAT5 to the β-casein probe although with a reduced efficiency. We obtained similar results concerning the involvement of tyrosine 343 in STAT5 activation using the BaF3 cell line. The implication of tyrosine 401 had not been tested but is highly likely since transfection of a F1-Y2-8 Epo-R mutant allowed full STAT5 activation by Epo in BaF3 cells (data not shown). Because Epo activates the high molecular mass forms of STAT5 in Epo-R transfected BaF3 cells (Pallard *et al.*, 1995b), both the long and short forms of STAT5 appear to be regulated by the same mechanisms. As previously reported by us (Gobert *et al.*, 1995) and by others (Klingmüller *et al.*, 1995; Yi *et al.*, 1995) attempts to demonstrate directly the phosphorylation of tyrosine 343 or tyrosine 401 were unsuccessful. However, tyrosine 343 was reported to be phosphorylated in Sf9 cells infected with an Epo-R encoding baculovirus (He *et al.*, 1993). The hypothesis that the Tyr343 to Phe343 replacement modifies the local conformation of a region required for STAT5 activation is of very low probability since this replacement is the most conservative and did not inhibit STAT5 activation provided that Tyr401 was present (F1-Y2, F1-Y2-3, F1-Y2-4 and F1-Y2-8 mutants). Moreover, the ability of tyrosine phosphorylated peptides corresponding to the sequences surrounding Tyr343 and Tyr401 to inhibit DNA binding to activated STAT5 strongly suggest that these sequences are recognized directly by the STAT5 SH2 domain. Thus, it is likely that these tyrosines are phosphorylated but that our detection methods are not sensitive enough to evidence directly this phosphorylation. We propose that the SH2 domain of STAT5 interacts with the phosphorylated tyrosines 343 and 401 of the Epo-R and that this interaction puts the STAT5 protein close to the Epo-R-associated JAK2 kinase that could efficiently phosphorylate it. The presence of multiple tyrosines able to mediate the phosphorylation/activation of STAT3 were shown in the LIF-R and in the gp130 chain of the IL-6-R. As in the case of the Epo-R, each of these sites alone is able to mediate full STAT activation (Stahl *et al.*, 1995). Moreover, the activation of STAT1 by IFN γ requires the tyrosine residue at position 440 in the cytoplasmic domain of the IFNγ receptor α subunit (Greenlund *et al.*, 1994).

The sequences surrounding Tyr343 and Tyr401 show some homology (DTY^{343}LVLD and FEY^{401}TILD) with an acidic residue at position –1 or –2 relative to the tyrosine residue, a hydrophobic residue at position +2, leucine and aspartic acid at positions 3 and 4 respectively. The sequence surrounding Tyr431 is more distantly related (YLY^{431}LVVS) with only hydrophobic residues in positions +2 and +3. Two tyrosines (Tyr392 and Tyr510) of the IL-2 receptor β chain have been shown to be necessary for full STAT5 activation by IL-2 (Lin *et al.*, 1995). The sequence surrounding Tyr510 of IL-2-R β chain (DAY^{510}LSLQ) exhibits a clear homology with the sequence surrounding Tyr343 of the Epo-R but that around Tyr392 (DAY^{392}CTFP) does not show evident homology with Epo-R sequences involved in STAT5 activation. The sequences required for STAT3 activation by gp130 or LIF-R have been determined. The presence of a glutamine residue at position +3 relative to the phosphorylated tyrosine appears to be necessary for STAT3 activation. A proline residue at position +2 was also often present in STAT3 activation sequences but its requirement seems to be less stringent (Stahl *et al.*, 1995). The tyrosine residues at positions 578 and 606 of the IL-4 receptor are necessary for the activation of STAT6 (Hou *et al.*, 1994). These two tyrosine residues are followed by a lysine at position +1 and a phenylalanine residue at position +3. Thus, receptor sequences involved in STAT3, STAT5 and STAT6 activation appear to be different and can explain the specific activation of a STAT by a cytokine.

Is STAT5 activation important for Epo-R mitogenic signalling?

We have previously shown that the removal of all tyrosine residues from the intracellular domain of the Epo-R decreases its ability to transduce a mitogenic signal. Expression and Epo stimulation of a higher number of ZERO (F1) Epo-R mutants than wild-type Epo-Rs are required for Epo-induced mitogenicity in BaF3 cells and in FDCP-1 cells. In contrast, BaF3 or FDCP-1 cells transfected with Epo-Rs containing only Tyr343 (Y1) exhibited a normal Epo sensitivity for cell proliferation (Gobert *et al.*, 1995). In this study, we observed a correlation between STAT5 activation and Epo sensitivity. FDCP-1 cells expressing receptors with low STAT5 activation potency (namely, F1, F1-Y3-4 and F1-Y5-8) exhibited the same decreased sensitivity towards the mitogenic signal as those previously reported for cells expressing the ZERO Epo-R mutant, despite the high levels of Epo binding sites expressed on their cell surface. The other Epo-R mutants tested in this study (Y1, F1-Y2, F1-Y2-3, F1-Y2-4 and F1-Y2-8) conferred STAT5 activation to transfected BaF3 or FDCP-1 cells and a mitogenic Epo sensitivity similar to that of the wild-type Epo-R (Figure 8; Gobert *et al.*, 1995 and unpublished results). These results suggest that STAT5 activation could be involved in cell proliferation. On the other hand, STAT5 activation by IL-2 involves a region of the IL-2 β chain dispensable for cell proliferation (Fuji *et al.*, 1995; Lin *et al.*, 1995) and it has been concluded that IL-2-stimulated STAT5 activation was not essential for IL-2 proliferative signal transduction in BaF3 cells (Fuji *et al.*, 1995). The redundancy of independent STAT5 activation sites in the Epo-R suggest that this transduction pathway could play a major role in Epo signalling. STAT5 dominant negative mutants will be necessary to assess directly the role of STAT5 in mitogenic signalling induced by Epo.

Materials and methods

Reagents

The monoclonal anti-phosphotyrosine antibody 4G10 was a generous gift of Dr B.Drucker. Chicken anti-STAT5 antibodies were produced as previously described (Wakao *et al.*, 1994). Anti-STAT1 antibodies were purchased from Transduction Laboratories; anti-STAT3, anti-STAT4 and anti-STAT6 antibodies were kindly provided by Dr D.Levy (New York University Medical Center), Dr J.Darnell (New York) and Dr S.McKnift (San Francisco) respectively. Highly purified recombinant human Epo (specific activity 200 000 U/mg) was a generous gift of Dr M.Brandt (Boehringer Mannheim) and recombinant murine IL-3 was from Pepro Tech Inc. Tyrosine phosphorylated and non-phosphorylated peptides were synthesized by Chiron Mimotopes (Lyon, France). All other reagents were purchased from Sigma.

DNA constructs and expression vectors

The intracellular domain of the murine Epo receptor contains eight tyrosines [positions 343 (Y1), 401 (Y2), 429 (Y3), 431 (Y4), 443 (Y5), 460 (Y6), 464 (Y7) and 479 (Y8)] of the mature protein (Figure 3). Mutation of tyrosine 343 was previously described (Gobert *et al.*, 1995). In order to remove all the tyrosines of the COOH end of the receptor (mutants F1 and Y1), the *Hin*dIII–*Cla*I fragment was excised and an adaptator with a stop codon and an *Eco*RI site was inserted. The other receptor mutants were produced from PCR-amplified fragments; for F1-Y2, F1-Y2-3 and F1-Y2-4 contructs, stop codons were added after codons 428, 430 and 442 respectively (i.e. just before tyrosines Y3, Y4 or Y5); for F1-Y3-4 and F1-Y5-8, a *Hin*dIII restriction site was created four amino acids before tyrosines Y3 and Y5 respectively. A stop codon was inserted just before the tyrosine Y5 for F1-Y3-4 and the stop codon of the wild-type receptor was used for F1-Y5-8. The amplified fragments were fully sequenced and ligated to the *Hin*dIII restriction site of the F1 receptor mutant. All the receptors were lastly cloned into a modified pCDNA3 expression vector where the CMV promoter and the neomycine resistance gene were changed respectively for a RSV promoter and a puromycine resistance gene.

Cell cultures

Non-transfected FDCP-1 cells were maintained in α minimum essential medium (α MEM) containing 10% fetal calf serum and 4% WEHI conditioned medium as a source of IL-3. After transfection and selection, Epo sensitive cells were maintained in α MEM supplemented with 10% FCS and 2 U/ml of Epo. For signal transduction experiments, the cells were grown using 5% WEHI conditioned medium as a source of growth factor and the cells were starved for 4 h by incubation in Iscove modification of Dulbecco's minimum essential medium containing 0.4% bovine serum albumin and 20 μg/ml iron saturated transferrin.

Stable transfections

FDCP-1 cells were transfected by electroporation using a Bio-Rad gene pulser set at 250 V and 960 μF. Fifty micrograms of plasmid were used in each transfection. After a 48 h incubation into IL-3 containing medium, the cells were selected for growing in the presence of 2 U/ml of Epo after removal of WEHI conditioned medium.

Receptor measurement

Epo was iodinated using Iodogen (Pierce) as previously described (Mayeux *et al.*, 1990) with specific radioactivities ranging from 30 to 60×10^6 c.p.m./μg. Epo binding experiments were carried out as previously described (Mayeux *et al.*, 1987, 1990, 1991).

Immunoprecipitation and Western blotting

Immunoprecipitations and Western blots were done as previously described (Dusanter-Fourt *et al.*, 1992). ECL (Amersham Ltd, Les Ullis, France) was used for the Western blot revelations.

Preparation of nuclear extracts

Starved cells were stimulated at 37°C with either Epo or IL-3 as described in Results and quickly chilled with ice-cold PBS. The cells were pelleted and solubilized with buffer A [buffer A: 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM Na_2VO_4, 0.2% Nonidet P40 (NP40), 10% glycerol and 1 μg/ml each of aprotinin, pepstatin and leupeptin, pH 7.9]. The lysates were centrifuged at 20 000 *g* for 2 min and the pellet was extracted with buffer B (buffer B: 20 mM HEPES, 350 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na_2VO_4, 20% glycerol and 1 μg/ml each of aprotinin, pepstatin and leupeptin, pH 7.9) using 1 ml of buffer B for 5×10^7 cells. The extracts were centrifuged at 20 000 *g* for 5 min and supernatants were quickly frozen and stored at –80°C.

Electrophoretic mobility shift assays (EMSA)

Most experiments reported in this paper used STAT5 binding site from the bovine β-caseine promoter (5′-AGATTTCTAGGAATTCAAATC-3′; 'β-casein probe') as a probe. Two microliters of nuclear extracts were mixed with 20 μl of binding buffer containing 60 000 c.p.m. end-labelled probe [binding buffer: 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP40, 5% glycerol, 1 mg/ml bovine serum albumin and 2 mg/ml poly(dI-dC) pH 7.5] and the mixture was incubated for 30 min at 4°C. Complexes were separated on 4 or 6% non-denaturating polyacrylamide gels in 0.25× TBE and detected by autoradiography. In some experiments, the radioactivity was quantified using a PhosphorImager (Molecular Dynamics model SI).

Cell proliferation measurements

Cell proliferation was measured using AlamarBlue (Interchim, Montluçon, France), a fluorescent analogue of 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT), following the manufacturer's instructions. Briefly, 5×10^3 cells were seeded in 96-well plates in the presence of increasing concentrations of Epo and incubated for 72 h at 37°C. Then, AlamarBlue was added and the cultures were incubated for 3 h before analysis using a plate fluorimeter with an excitation wavelength of 560 nm and an emission recording wavelength of 590 nm.

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