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# Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways

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**Signal transducers and activators of transcription (STAT) proteins play an important role in cytokine signal transduction in conjunction with Janus kinases (JAKs). MGF/STAT5 is known as prolactin regulated STAT. Here we demonstrate that interleukin 2 (IL-2) as well as erythropoietin (EPO) stimulate STAT5 and induce tyrosine phosphorylation of STAT5. These IL-2- and EPO-induced STATs have an identical DNA binding specificity and immunoreactivity. We also show that IL-4 induces a DNA binding factor which possesses similar, but distinct, DNA binding specificity from that of STAT5 and is immunologically different from STAT5. Analysis of two EPO receptor (EPOR) transfected CTLL-2 cell lines discloses that IL-2 activates JAK1 and JAK3 as well as STAT5, while EPO stimulates STAT5 and JAK2 in EPO-responsive CTLL-2 cells (ERT/E2). On the contrary, EPO activates neither JAK2 nor STAT5 in other cell lines that failed to respond to EPO (ERT cells). EPOR and JAK2 associate with each other regardless of EPO presence in ERT/E2 cells, however, such an interaction is not present in ERT cells. Thus, EPOR and JAK2 association seems to be important for EPO responsiveness in CTLL-2 cells.**

*Key words*: erythropoietin/interleukin 2/Janus kinases (JAK)/signal transduction/STAT5

## Introduction

Although cytokine receptors lack any intrinsic enzymatic activity such as protein tyrosine kinases (PTK), cytokines rapidly induce tyrosine phosphorylation of cellular proteins, indicating a role for PTK(s) in their signaling (for reviews, see Miyajima *et al.*, 1992, 1993). In fact, several Src-like PTKs are activated by certain cytokines (Torigoe *et al.*, 1992; Kobayashi *et al.*, 1993). Recently, a distinct subfamily of PTKs known as Janus kinases (JAKs) was found to play an important role in cytokine signaling (for reviews, see Darnell *et al.*, 1994; Ihle *et al.*, 1994). This JAK-mediated signaling pathway, originally found in the interferon (IFN) system, is now believed to be shared by various cytokines. This signaling system is unique for its direct link of the receptor–ligand interaction to gene expression in the nucleus. JAKs associate with cytokine receptors and are stimulated when cytokines bind to their cognate receptors. The activated JAKs in turn convert cryptic cytoplasmic transcription factors, known as signal transducers and activators of transcription (STATs), into active forms by tyrosine phosphorylation. The tyrosine-phosphorylated STATs form homodimers or heteromers and translocate into the nucleus, where they bind to their specific target sequences and control gene expression. At present, four kinds of JAKs (JAK1, 2, 3 and Tyk2), and eight different STATs (STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5A, STAT5B and IL-4STAT) have been molecularly cloned (Akira *et al.*, 1994; Darnell *et al.*, 1994; Hou *et al.*, 1994; Ihle *et al.*, 1994; Wakao *et al.*, 1994; Yamamoto *et al.*, 1994; Zhong *et al.*, 1994a,b; Mui *et al.*, 1995). Since each cytokine activates a specific set of JAKs and STATs, this signaling pathway may be relevant to the specific function of each cytokine.

Erythropoietin (EPO) and interleukin 2 (IL-2) stimulate the proliferation of erythroid and T cells, respectively. The EPO receptor (EPOR) belongs to the cytokine receptor superfamily and functions as a homodimer (Watowich *et al.*, 1992). The high affinity IL-2 receptor (IL-2R) consists of α, β and γ chains; the β and γ chains, both essential for signal transduction, are also members of the cytokine receptor superfamily (Takeshita *et al.*, 1992; Taniguchi and Minami, 1993). While EPO and IL-2 stimulate the proliferation of different cell types, they induce similar intracellular responses such as activation of Ras, Raf and Map kinase as well as expression of c-*fos*, c-*myc* and *pim-1* genes (Carroll *et al.*, 1991; Satoh *et al.*, 1991; Lilly *et al.*, 1992; Maslinski *et al.*, 1992; Torti *et al.*, 1992; Beadling *et al.*, 1993; Miura *et al.*, 1994b). Concomitantly, the EPOR possesses a critical cytoplasmic domain for signaling, which is similar to the serine-rich region of IL-2Rβ (D'Andrea *et al.*, 1989). Accordingly, EPO and IL-2 equally support the proliferation of IL-3-dependent cells when their receptors are ectopically expressed (Hatakeyama *et al.*, 1989; Jones *et al.*, 1990; Li *et al.*, 1990; Yoshimura *et al.*, 1990; Chiba *et al.*, 1993; Sakamaki *et al.*, 1993). These results suggest that there might be a common component(s) shared by EPO, IL-2 and IL-3 in their signaling. However, inconsistent results have accumulated as to the function of EPOR in an IL-2-dependent T-cell line, CTLL-2, transfected with EPOR cDNA. It has been shown that EPO induces tyrosine phosphorylation and supports the growth of such a T-cell transfectant (Showers *et al.*, 1992; Barber and D'Andrea, 1994). In contrast, Yamamura *et al.* (1992, 1994) reported that EPO neither induces tyrosine phosphorylation nor supports proliferation of their CTLL-2 transfectants expressing EPOR. Interestingly, overexpression of Kirsten Ras results in EPO responsiveness in such a cell line and this conversion is accompanied by the appearance of a 160 kDa cell surface protein co-immunoprecipitated with EPOR (Yamamura *et al.*, 1992, 1994). We also independently generated CTLL-2 transfectants expressing

EPOR and found that they did not proliferate in response to EPO immediately. However, after a latency of ~1 week, EPO-responsive cells emerged from EPO non-responsive CTLL-2 transfectants (Sakamaki *et al.*, 1993). Thus, the discrepancy among different investigators is likely to be due to the difference in CTLL-2 transfectant cells used for each experiment and also suggests the difference in the activation mechanism between EPOR and IL-2R. In fact, recently it has been reported that EPO activates JAK2, whereas IL-2 activates JAK1 and JAK3 (Witthuhn *et al.*, 1993, 1994; Johnston *et al.*, 1994). The involvement of JAK family members in IL-2 and EPO signaling strongly implicated STATs in their signaling pathways.

In this paper we demonstrate that EPO, as well as IL-2, activates STAT5, which was originally identified as mammary gland factor (MGF) regulated by prolactin (Wakao *et al.*, 1994). STAT5 was recently found to be activated by IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Mui *et al.*, 1995). We also show that IL-2 stimulates JAK1 and JAK3 while EPO activates JAK2 in EPO-responsive CTLL-2 transfectant cells and present evidence that EPOR associates with JAK2 regardless of the EPO stimulation in such an EPO-responsive cell line.

## Results

### *Activation of STAT5 upon IL-2 challenge*

The fact that certain cytotoxic T-lymphocytes (CTL) express some milk proteins, including β-casein (Grusby *et al.*, 1990), and that STAT5 is expressed in the thymus (Wakao *et al.*, 1994), prompted us to investigate whether STAT5 is involved in IL-2 signaling. To test this hypothesis, electrophoretic mobility shift assay (EMSA) was performed using a prolactin-responsive element (PRE) as a probe (Wakao *et al.*, 1994). CTLL-2 is an IL-2-dependent murine cytotoxic T-cell line which also responds to IL-4. After stimulation with either IL-2 or IL-4, specific DNA binding proteins appeared (Figure 1A and B, lanes 2–6, arrows), whereas no DNA binding activity was detected in the absence of these cytokines (Figure 1A and B, lanes 1). DNA binding activity reached its maximum at 40 min and 20 min after IL-2 and IL-4 stimulation, respectively, then gradually declined (Figure 1A and B). STAT5-specific polyclonal antibodies were used to examine the presence of STAT5 molecule in these complexes. Both anti-STAT5 antibodies N1 and N5, which were raised against the N- and C-terminal portions, respectively, of ovine STAT5, supershifted the IL-2-induced factor (Figure 2A, lanes 3 and 4), whereas pre-immune serum neither supershifted nor inhibited the DNA binding of IL-2- and IL-4-induced factors (Figure 2A, B, lanes 2). Intriguingly, N5 slightly supershifted the IL-4-induced complex, while N1 failed to bind to this complex (Figure 2B, lanes 3 and 4). Similar results were obtained with another mouse T-cell line HT2 (H.W., unpublished results).

DNA binding specificity of each factor was further assessed by competition experiments using a variety of oligonucleotides such as PRE, mutated PRE, IL-4 NAF binding sites (Kotanides and Reich, 1993), IL-6 response element, IFN-γ activation sequences (GAS), c-*sis* inducible element (SIE) and interferon-stimulated response element (ISRE). An excess amount of the unlabeled wild-type

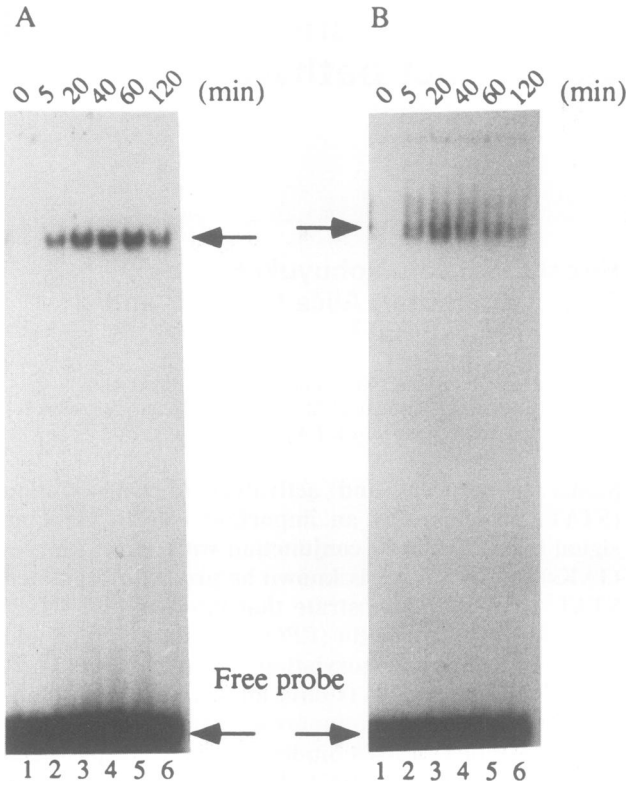


**Fig. 1.** IL-2 and IL-4 induce a DNA binding protein that recognizes the prolactin response element (PRE). After IL-2 starvation, CTLL-2 ($2\times10^7$/experiment) was treated with mouse IL-2 (10 ng/ml) (**A**, lanes 2–6) or with mouse IL-4 (10 ng/ml) (**B**, lanes 2–6) or left untreated (A, B, lanes 1) for the indicated time and nuclear extracts were used for electrophoretic mobility shift assays (EMSA) using a PRE of the β-casein promoter as a probe. The DNA–protein complex induced by IL-2 and IL-4 is indicated by arrows and the position of the free probe is also shown.

PRE completely abolished the DNA binding of both IL-2- and IL-4-induced factors, whereas the mutated PRE failed to abrogate the binding (Figure 2A and B, lanes 5 and 6). IL-4 NAF binding sites such as interferon regulatory factor 1 (IRF1), FcεRIIb, FcγRI, as well as acute phase response element (APRE), which is an IL-6 response element (Hattori *et al.*, 1990), inhibited the binding of both IL-2- and IL-4-induced factors (Figure 2A and B, lanes 7–10). Neither Ly6E, SIE nor ISRE, which are binding sites of STAT1, STAT2 and STAT3, interfered with binding (Figure 2A and B, lanes 12, 14 and 15). However, although the GAS of guanylate binding protein (GBP) weakly inhibited the DNA binding of the IL-4-activated factor, it had little effect on the binding of the IL-2-activated factor (Figure 2A and B, lanes 13). Furthermore, Iε, an IL-4 response-like element in the promoter of the murine Ig heavy chain constant region, revealed the difference in DNA binding specificity between IL-2- and IL-4-activated factors, i.e. the binding of the IL-4-activated factor was completely abolished by the Iε oligonucleotide, while the binding of the IL-2-activated factor was only slightly inhibited (Figure 2A and B, lanes 11). The DNA binding specificity of the IL-2-activated factor was identical to that of STAT5 induced by prolactin, IL-3, IL-5 and GM-CSF (Gouilleux *et al.*, submitted; Mui *et al.*, 1995). These results indicated that IL-2 activates

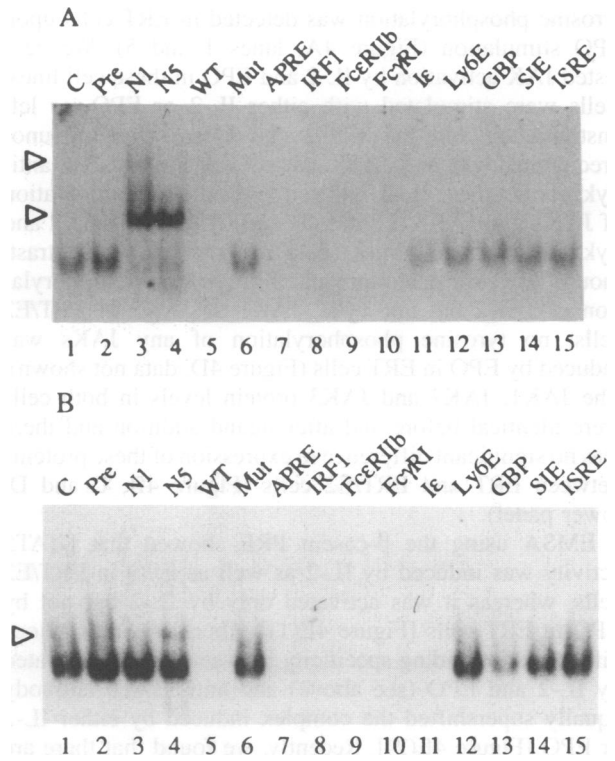


**Fig. 2.** IL-2 activates STAT5 while IL-4 induces a factor possessing a similar but not identical DNA binding specificity as that of STAT5. (**A**) STAT5 induction upon IL-2 challenge. Nuclear extracts from CTLL-2 cells stimulated with IL-2 for 20 min were subjected to EMSA. Control (lane 1). Pre-immune STAT5-IgY, N1-IgY or N5-IgY were added to the binding reaction (lanes 2, 3 and 4). Supershifted bands are shown by triangles. Competition experiments were performed in the presence of a 100-fold molar excess of the indicated oligodeoxynucleotides (lanes 5–15). These are double-stranded wild-type PRE (5′-AGATTTCTAGGAATTCAATCC-3′) (lane 5), mutated PRE (5′-AGATTTATTTTAATTCAATCC-3′) (lane 6), core APRF (5′-GATCCTTCTGGGAATTCCTA-3′) (lane 7), IRF1 (5′-GATCC-ATTTCCCCGAAATGA-3′) (lane 8), FcεRIIb (5′-TCCCTTTCTT-AGAAATTC-3′) (lane 9), FcγRI (5′-GTATTTCCCAGAAAAGG-AAC-3′) (lane 10), Iε (5′-GTCAACTTCCCAAGAACAGAA-3′) (lane 11), Ly6/E (5′-CATGTTATGCATATTCCTGTAAGTG-3′) (lane 12), GBP (5′-AAGTACTTTCAGTTTCATATTACTCTAAATC-3′) (lane 13), SIE (5′-GTCGACAGTTCCCGTCAATC-3′) (lane 14) and ISRE (5′-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3′) (lane 15). (**B**) IL-4 induces a factor that recognizes PRE. EMSA was performed as described in (A) except CTLL-2 cell extracts treated with murine IL-4 for 20 min were used. All treatments were the same as in (A).

STAT5 while IL-4 stimulates another protein which possesses a similar but different binding specificity from that of STAT5 and is immunologically different from STAT5. Stable transfection of STAT5 cDNAs into ERT/E2 cells confirmed this (see below, Figure 4F)

### *Activation of JAK2 and STAT5 by EPO*

EPO induces tyrosine phosphorylation of several proteins including EPOR, Shc, protein tyrosine phosphatase (PTP) and JAK2 (Damen *et al.*, 1993; Witthuhn *et al.*, 1993). Using an EPO-responsive human erythroleukemia cell line TF-1 (Kitamura *et al.*, 1989), we confirmed that although TF-1 cells express JAK1, JAK2, JAK3 and Tyk2, EPO specifically tyrosine phosphorylated JAK2 (data not shown). We then searched for a STAT which is activated by EPO. The observation that STAT5 is expressed ubiquitously in various tissues and cells, and that IL-3, IL-5 and GM-CSF, all of which stimulate JAK2, also induce a similar signal to that of EPO strongly suggested the possibility of STAT5 involvement in the EPO signaling pathway (Wakao *et al.*, 1994; Mui *et al.*, 1995).

To test this supposition, EMSA was performed with nuclear extracts from TF-1 cells either stimulated or unstimulated with EPO using PRE as a probe. EPO induced a specific DNA binding protein (Figure 3A, lane 1 and 3B, lanes 1–6) and this DNA–protein complex was supershifted by anti-STAT5 antibodies (Figure 3A, lanes 3 and 4) but not by the pre-immune serum (Figure 3A, lane 2). The DNA binding specificity of this complex was evaluated further by competition experiments using different oligodeoxynucleotides as mentioned above and showed the same profile as that induced by IL-2 (Figure 2A, Figure 3A, lanes 5–15). These data strongly implied that EPO also activates STAT5. In fact, results from the stable transfectants expressing the cloned STAT5 cDNA supported this idea (see below, Figure 4F).

It has been shown *in vitro* that STAT5 is a substrate of JAK2 (Gouilleux *et al.*, 1994). We next studied the kinetics of STAT5 and JAK2 activation to see whether there is a correlation between JAK2 and STAT5 stimulation in TF-1 cells. To evaluate the activation of these proteins, EMSA was employed for STAT5 activation and tyrosine phosphorylation was examined for JAK2 activation (Figure 3B). In the absence of EPO, neither STAT5 stimulation nor JAK2 tyrosine phosphorylation was detected (Figure 3B, lane 1, upper and middle panels). STAT5 activation was observed 5 min after the EPO challenge and lasted for at least 3 h (Figure 3B, upper panel). This coincided well with the tyrosine phosphorylation kinetics of JAK2 (Figure 3B, middle panel). The JAK2 protein level was constant throughout the experiments (Figure 3B, lower panel). We also observed that STAT5 was tyrosine phosphorylated upon IL-2 or EPO challenge in CTLL-2 or TF-1 cells, respectively (Figure 3C and D), consistent with the data that STATs are tyrosine phosphorylated upon cytokine binding to the cognate receptor. These results indicate that JAK2 also utilizes STAT5 as a substrate in EPO signaling.

### *Activation of JAKs and STATs by IL-2 and EPO in EPOR-transfected CTLL-2 cells*

EPO, IL-3, GM-CSF, IL-5 and PRL all activate JAK2 (for review, see Ihle *et al.*, 1994), whereas IL-2 activates JAK1 and JAK3 but not JAK2 (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994). Nevertheless, all these cytokines stimulate STAT5. This raises the question as to how STAT5 is activated. To address this problem, we used CTLL-2 transfectants that expressed EPOR. As described above, we previously established an IL-2-dependent CTLL-2 transfectant expressing EPOR (ERT). ERT cells were unresponsive to EPO but proliferated in response to IL-2. EPO-responsive sublines (ERT/E) were readily and reproducibly obtained by incubating ERT cells with EPO. ERT/E2 is one such subline that proliferated in response to either IL-2 or EPO. To exclude the possibility that the differing EPO responsiveness merely reflects differences in EPOR expression and/or in affinity for EPO, the EPOR number and the $K_d$ for EPO binding in ERT and ERT/E2 cells were determined. The number of EPOR in ERT and

ERTR/E2 cells was 1300 and 1900 per cell, respectively, and the $K_d$ values for EPO in ERT and ERT/E2 cells were 2.2 nM and 1.3 nM. This similarity indicates that some intracellular components may mediate EPO responsiveness.

To find out the difference between these two cell lines, we analyzed the protein tyrosine phosphorylation of whole cell lysates, tyrosine phosphorylation of JAKs and activation of STAT5 by EPO and IL-2. Figure 4A shows the total tyrosine phosphorylation of ERT and ERT/E2 cells before and after EPO or IL-2 challenge. In the absence of ligand, ERT and ERT/E2 cells exhibited the same basal level of phosphorylation (Figure 4A, lanes 1 and 2). IL-2 induced tyrosine phosphorylation of the four distinct proteins (indicated by arrows) in both ERT and ERT/E2 cells (Figure 4A, lanes 3 and 4). EPO challenge resulted in tyrosine phosphorylation of five additional proteins in ERT/E2 cells (Figure 4A, lane 6). In contrast, no induced tyrosine phosphorylation was detected in ERT cells upon EPO stimulation (Figure 4A, lanes 1 and 5). We next tested JAK activation by IL-2 and EPO in these cell lines. Cells were stimulated with either IL-2 or EPO, or left unstimulated and the whole cell lysate was immunoprecipitated with anti-JAK1, anti-JAK2, anti-JAK3 or anti-Tyk2 antibodies. IL-2 induced tyrosine phosphorylation of JAK1 and JAK3 (Figure 4B and C), but not JAK2 and Tyk2, in both cell lines (data not shown). In contrast, though EPO stimulation resulted in tyrosine phosphorylation of JAK2 but not Tyk2, JAK1 or JAK3 in ERT/E2 cells, no tyrosine phosphorylation of any JAKs was induced by EPO in ERT cells (Figure 4D, data not shown). The JAK1, JAK2 and JAK3 protein levels in both cells were identical before and after ligand addition and there was no significant difference in expression of these proteins between ERT and ERT/E2 cells (Figure 4B, C and D, lower panel).

EMSA using the β-casein PRE showed that STAT5 activity was induced by IL-2 as well as EPO in ERT/E2 cells, whereas it was activated only by IL-2 but not by EPO in ERT cells [Figure 4E(1)]. There was no obvious difference in binding specificity between STAT5 activated by IL-2 and EPO (see above) and anti-STAT5 antibody equally supershifted the complex induced by either IL-2 or EPO [Figure 4E(2)]. Recently, we found that there are two distinct STAT5 genes (STAT5A and STAT5B) in the mouse. These two STAT5s are highly homologous to ovine STAT5/MGF (95 and 92% identical at the amino acid level) and both are activated by IL-3, IL-5 and GM-CSF in IL-3-dependent myeloid cell lines as well as in COS7 cells transiently transfected with STAT5 and the receptor cDNAs (Mui *et al.*, 1995). To confirm that both STAT5s are activated by IL-2 and EPO, we established ERT/E2 cell lines stably transfected with either murine STAT5A or STAT5B. To distinguish these exogenous STAT5s from the endogenous ones, an epitope tag

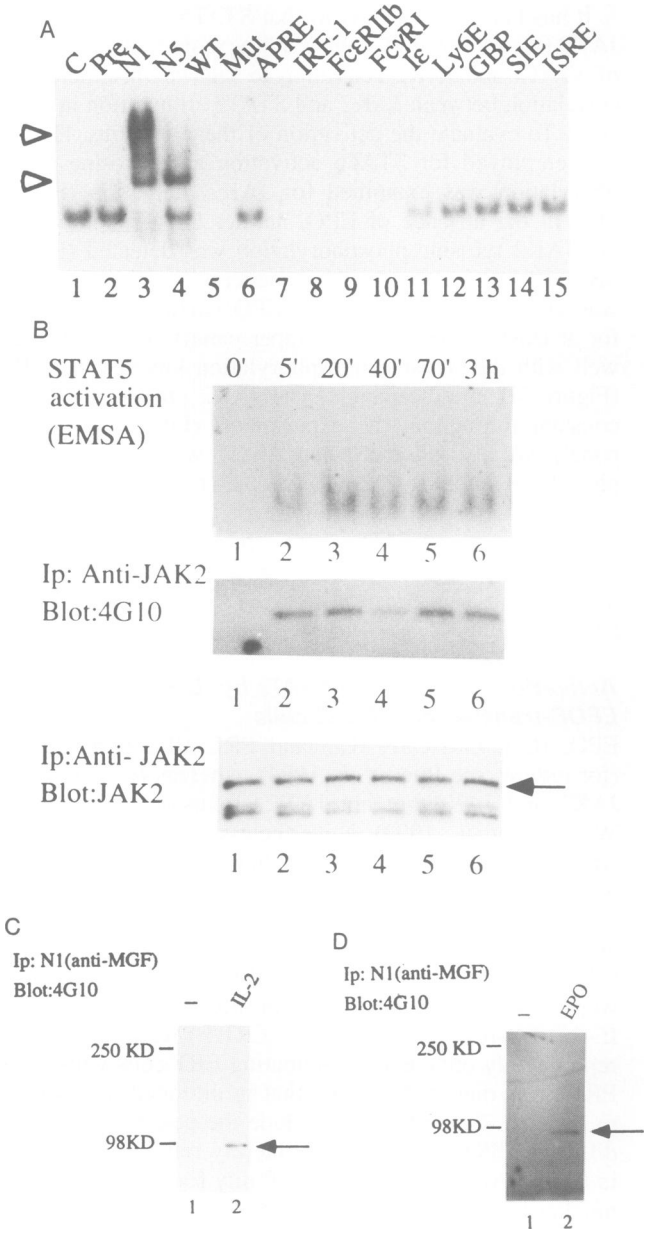


**Fig. 3.** STAT5 and JAK2 induction upon EPO stimulation in TF-1. (**A**) STAT5 induction upon EPO challenge. After starvation without any growth factor, TF-1 cells ($2\times10^7$) were treated with EPO (5 U/ml) for 20 min and nuclear extracts were prepared. EMSA was carried out as described in Figure 1. All antibody treatments and competition experiments were the same as in Figure 2. Supershifted bands are indicated by triangles. (**B**) STAT5 and JAK2 activation kinetics upon EPO challenge. Nuclear extracts were prepared at the indicated times ($2\times10^7$cells/experiment) after EPO stimulation and EMSA was performed as described above (upper panel). JAK2 was immunoprecipitated with anti-JAK2 antibody (UBI) from TF-1 cells at the indicated times after EPO challenge, separated on SDS–PAGE, then probed with anti-phosphotyrosine antibody 4G10 (middle panel). The same blot was reprobed with anti-JAK2 antibody to evaluate the protein level. The band which corresponds to tyrosine-phosphorylated JAK2 is shown by an arrow (lower panel). (**C**) IL-2 induces tyrosine phosphorylation of STAT5. CTLL-2 cells ($2\times10^7$) were starved without IL-2 for 6 h and challenged with mouse IL-2 (10 ng/ml) for 20 min. Cells were lysed with Triton X-100 as described and immunoprecipitated with N1-IgY. Precipitated proteins were separated on SDS–PAGE and probed with 4G10. Tyrosine-phosphorylated protein is indicated by an arrow. Non-stimulated (lane 1), stimulated with IL-2 (lane 2). (**D**) EPO induces tyrosine phosphorylation of STAT5. TF-1 cells ($4\times10^7$) were starved for 24 h without growth factor and then stimulated with EPO (5 U/ml) for 20 min. Cells were lysed, immunoprecipitated with N1-IgY, separated and probed with 4G10 as above. A protein detected by 4G10 is shown by an arrow. No addition of EPO (lane 1), EPO challenged (lane 2).

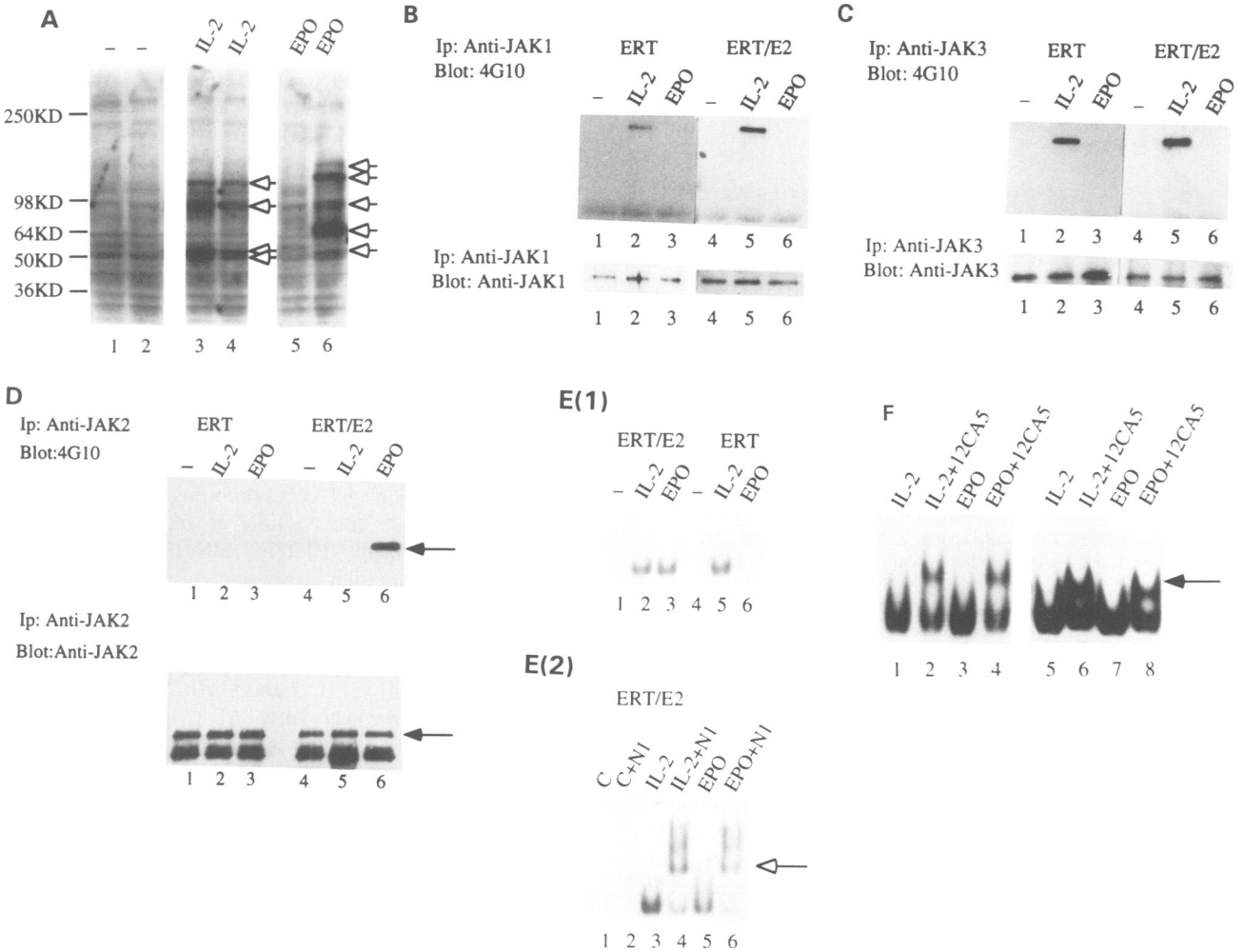


**Fig. 4.** Activation of STAT5 and different JAKs in ERT and ERT/E2 cells. (**A**) Total tyrosine phosphorylation induced by IL-2 and EPO in ERT and ERT/E2 cells. After starvation of ERT and ERT/E2 cells without IL-2 or EPO, cells ($4\times10^5$ cells per experiment) were stimulated with IL-2 (10 ng/ml) or EPO (5 U/ml) for 20 min, lysed, and proteins were separated by SDS–PAGE then blotted with anti-phosphotyrosine antibody 4G10. No stimulation (lanes 1 and 2), IL-2 stimulation, (lanes 3 and 4) and EPO stimulation (lanes 5 and 6) for ERT (lanes 1, 3 and 5) and for ERT/E2 cells (lanes 2, 4 and 6). Newly induced tyrosine-phosphorylated proteins are shown by arrows. (**B** and **C**) JAK1 and JAK3 activation in ERT and ERT/E2. After starvation, ERT and ERT/E2 cells ($2\times10^7$ for each experiment) were stimulated with IL-2 (10 ng/ml) or EPO (5 U/ml) for 20 min, lysed and immunoprecipitated with either anti-JAK1 (B) or anti-JAK3 antibody (C), separated by SDS–PAGE and probed with 4G10. Protein expression of JAK1 and JAK3 was verified by Western blotting (lower panels). Non-induced fraction (lanes 1 and 4), IL-2-induced fraction (lanes 2 and 5) and EPO-induced fraction (lanes 3 and 6). ERT cells (lanes 1–3), ERT/E2 cells (lanes 4–6). (**D**) JAK2 activation in ERT and ERT/E2 cells. Cells were stimulated and lysed as described above, followed by immunoprecipitation with anti-JAK2 antibody. After separation by SDS–PAGE, 4G10 was used to detect tyrosine-phosphorylated protein (upper panel). The blot was reprobed with anti-JAK2 antibody to evaluate the amount of protein (lower panel). Unstimulated (lanes 1 and 4), IL-2-stimulated (lanes 2 and 5) and stimulated with EPO (lanes 3 and 6). ERT cells (lanes 1–3), ERT/E2 cells (lanes 4–6). (**E**) STAT5 activation in ERT and ERT/E2 upon IL-2 and EPO challenge (1). After IL-2 or EPO starvation, ERT and ERT/E2 were challenged with either IL-2 or EPO and nuclear extracts were prepared and subjected to EMSA. Untreated (lanes 1 and 4); IL-2-treated (lanes 2 and 5); EPO-treated (lanes 3 and 6). ERT (lanes 4–6), ERT/E2 (lanes 1–3). Anti-ovine STAT5 antibody recognizes IL-2- and EPO-induced STAT5 (2). IL-2- and EPO-stimulated as well as non-stimulated extracts from ERT/E2 cells were used for EMSA with N1 antibody (lanes 2, 4 and 6) and without antibody (lanes 1, 3 and 5). Unstimulated (lanes 1 and 2), IL-2-induced (lanes 3 and 4) and EPO-stimulated (lanes 5 and 6). Supershifted bands are indicated by an arrow. (**F**) Murine STAT5A and B stably transfected into ERT/E2 cells are activated upon IL-2 and EPO stimulation. ERT/E2 cells were transfected stably with the tagged murine STAT5A and B separately and nuclear extracts were prepared and used for EMSA. The IL-2-induced fraction is shown in lanes 1, 2, 5 and 6 and the EPO-induced fraction in lanes 3, 4, 7 and 8. Monoclonal antibody against influenza tag (12CA5) was added in the binding reaction (lanes 2, 4, 6 and 8). STAT5A (lanes 1–4), STAT5B (lanes 5–8). The supershifted bands are shown by an arrow.

(influenza hemagglutinin) was attached to the N-terminus of both STAT5s (Munro and Pelham, 1984; Gouilleux *et al.*, 1994) and expressed individually in ERT/E2 cells. The stably transfected cells were then analyzed by EMSA. The DNA–protein complexes that appeared in response to either IL-2 or EPO (Figure 4F, lanes 1, 3, 5 and 7) were supershifted by the addition of monoclonal antibody against the epitope tag (12CA5) in both transfectants (Figure 4F, lanes 2, 4, 6 and 8). These data unequivocally demonstrated that IL-2 and EPO have the capacity to activate both STAT5A and STAT5B in T cells.

### *Association between EPOR and JAK2*

These results indicated that although both ERT and ERT/E2 cells were competent for STAT5 activation as well as JAK1 and JAK3 phosphorylation upon IL-2 challenge,

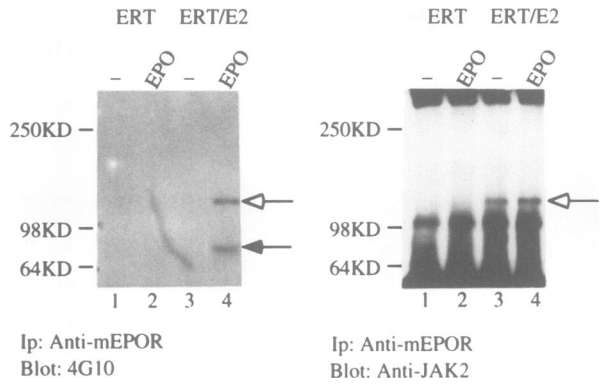


**Fig. 5.** JAK2 associates with EPOR in ERT/E2 but not in ERT cells regardless of the presence of EPO. EPOR was immunoprecipitated with anti-murine EPOR antibody from ERT/E2 and ERT cells ($5\times10^7$ cells for each experiment) with or without EPO in the presence of 1% digitonin instead of Triton X-100. After separation by SDS–PAGE, 4G10 was used to detect tyrosine-phosphorylated proteins. Probed with 4G10 (left panel). ERT cells are shown in lanes 1 and 2 and ERT/E2 cells in lanes 3 and 4; non-stimulated (lanes 1 and 3), EPO-induced (lanes 2 and 4). Tyrosine-phosphorylated 130 kDa protein as well as EPOR are shown by an open-headed and closed-headed arrow, respectively. The same blot was reprobed with anti-JAK2 antibody (right panel). ERT cells are shown in lanes 1 and 2 and ERT/E2 cells in lanes 3 and 4. JAK2 protein revealed after the anti-JAK2 antibody blotting is indicated by an arrow.

EPO failed to activate both JAK2 and STAT5 in ERT cells. Since there is no significant difference in the number of EPOR or in affinity for EPO between ERT and ERT/E2 cells, we compared the JAK2 protein expression level in both cell lines. As shown in Figure 4D, ERT and ERT/E2 cells expressed similar amounts of immunoprecipitated JAK2 protein. An equivalent amount of JAK2 protein was also detected when whole cell lysates were analyzed directly (data not shown). These observations suggested that there might be a defect in the EPOR–JAK2 interaction in ERT cells. Since EPOR associates with JAK2 physically without EPO stimulation (Witthuhn *et al.*, 1993), we examined the EPOR–JAK2 interaction in ERT and in ERT/E2 cells. A mild detergent, digitonin, was used instead of Triton X-100 in these experiments to visualize such an interaction (Miura *et al.*, 1994a). EPOR was immunoprecipitated with anti-EPOR from cells stimulated with or without EPO, and blotted with anti-phosphotyrosine as well as anti-JAK2 antibodies (Figure 5). EPO induced tyrosine phosphorylation of the 72 kDa EPOR as well as a 130 kDa protein in ERT/E2 cells (Figure 5, left panel, lane 4, arrows). Subsequent anti-JAK2 antibody blotting revealed that this 130 kDa tyrosine-phosphorylated protein was JAK2 (Figure 5, right panel, lane 4, arrow). In accordance with the previous report, JAK2 was co-immunoprecipitated with EPOR in ERT/E2 cells even in the absence of EPO (Figure 5, right panel, lane 3, arrow). In contrast, no tyrosine-phosphorylated protein was found in ERT cells regardless of EPO stimulation (Figure 5, left panel, lanes 1 and 2). Furthermore, JAK2 was not found to associate with EPOR in ERT cells regardless of ligand binding (Figure 5, right panel, lanes 1 and 2). When cell lysate was immunoprecipitated with anti-JAK2 antibody and blotted with 4G10, EPO challenge induced tyrosine phosphorylation of a 70 kDa protein as well as of JAK2 itself only in ERT/E2 cells. Subsequent probing with anti-EPOR antibody showed that this 70 kDa protein was EPOR (data not shown). These results demonstrated that EPOR failed to interact with JAK2 in ERT cells and the conversion to EPO responsiveness was accompanied by the association of EPOR and JAK2 in ERT/E2 cells.

## Discussion

### *STAT5 activation by IL-2 and EPO*

While IL-2 and EPO are known to activate JAK(s), the downstream molecule has not been identified. Here, we show that STAT5, originally characterized as MGF, whose activity is regulated by prolactin in the mammary gland (Wakao *et al.*, 1994), is also activated by IL-2 and EPO. This was shown by the identical DNA binding specificity of the EPO- and IL-2-activated factors, their cross-reactivity to the ovine STAT5 antibodies (Figures 2A and 3A) and the stable transfection of the epitope-tagged STAT5A or STAT5B into ERT/E2 cells (Figure 4F). STATs are known to bind to their target sequence as a homodimer or heteromer (Darnell *et al.*, 1994). STAT5A and STAT5B may form either homodimers or heterodimers upon stimulation with IL-3 or GM-CSF. STAT5A/B–DNA complexes induced by IL-3, IL-5 and GM-CSF do not appear to contain any other known STAT (Mui *et al.*, 1995). However, the possibility that STAT5 dimerizes with other STATs cannot be excluded. This could occur in specific cells or upon particular stimulation. Whether IL-2- or EPO-activated STAT5 forms a complex with other STATs remains to be examined.

IL-4-induced factor and STAT5 possess similar but distinct DNA binding specificities (Figure 2B). STAT5 recognizes 5′-TTCNNNGAA-3′ and the presence of three nucleotides between TTC and GAA appears to be critical for the optimal binding (Schmitt-Ney *et al.*, 1991; Wakao *et al.*, 1994; Gouilleux *et al.*, submitted). All the oligonucleotides that abrogated the binding of STAT5 to PRE conform to this criterion, however, the Iε site (TTCCCAAGAA) has four nucleotides between 5′-TTC and GAA-3′, and was ineffective for STAT5 binding. Recently, IL-4 STAT was purified using the FcγRI sequence (one of the STAT5 binding sequences) and the corresponding cDNA was isolated. The similarity between the IL-4 STAT and STAT5 protein is 34%. These proteins are the most homologous among STATs so far identified (Hou *et al.*, 1994). This may be consistent with our observation that the N5 antibody, which was raised against the SH2 domain of the ovine STAT5, poorly recognized the IL-4-activated factor (Figure 2B, lane 4). However, it is not yet clear whether this IL-4-activated factor in CTLL-2 cells is a mouse counterpart of the cloned IL-4 STAT.

Although IL-2R and IL-4R share the common γ subunit for their receptors (Kondo *et al.*, 1993; Russell *et al.*, 1993), our current data indicate that they do not employ the same STAT. This is an intriguing contrast to other receptor systems in which a shared receptor subunit is used for their signaling, i.e. the common β subunit for the GM-CSF, IL-3 and IL-5 receptors and gp130 for IL-6, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and oncostatin M (OSM) receptors. GM-CSF, IL-3 and IL-5, which possess similar biological effects, induce similar intracellular signals including the

stimulation of STAT5s (Mui *et al.*, 1995). Likewise, IL-6, LIF, CNTF and OSM, that exhibit similar functions, activate STAT3 (Akira *et al.*, 1994). In contrast, signaling by IL-2 and IL-4 which share the common γ subunit, differs in the induction of tyrosine phosphorylation, in their ability to activate the Ras signaling pathway and in the use of STAT (Satoh *et al.*, 1991; Wang *et al.*, 1992). Thus, the functional significance of the γ chain in IL-2, IL-4, IL-7, IL-9, IL-15 and, probably, IL-13 signaling is different from that of common β and gp130. The use of the different STATs in IL-2 and IL-4 signaling may reflect, in part, their unique signaling which eventually leads to distinct biological consequences elicited by these cytokines.

The fact that prolactin, IL-2, EPO, as well as IL-3, IL-5 and GM-CSF activate STAT5 strongly implies that STAT5 may have a fundamental role in the signal transduction by these cytokines. Identification of genes regulated by STAT5 should shed light on this question.

### *How is STAT5 activated by EPO and IL-2?*

GM-CSF, IL-3 and IL-5 induce activation of JAK2, STAT5A and STAT5B (Ihle *et al.*, 1994; Mui *et al.*, 1995). Likewise, the present study demonstrates that EPO stimulates JAK2 and STAT5 in TF-1 cells (Figure 3A and B) and in a T-cell transfectant expressing EPOR (ERT/E2) (Figure 4D and E). Similar results were obtained in mouse erythroleukemic cell lines as well as in BaF3 cells transfected with EPOR (H.W., unpublished). Intriguingly, although both EPO and IL-2 activated STAT5A and STAT5B, EPO tyrosine phosphorylates exclusively JAK2, while IL-2 tyrosine phosphorylates JAK1 and JAK3 but not JAK2 (Figure 4B, C and D). These data indicate that any of JAK1, JAK2 and JAK3 can tyrosine phosphorylate STAT5A and STAT5B. Whether JAK1 and/or JAK3 directly phosphorylates STAT5 has yet to be determined. The tyrosine residue at position 694 in STAT5 is required for induction of its DNA binding ability by EPO stimulation (Gouilleux *et al.*, submitted). Thus, identification of the tyrosine residue(s) in STAT5 upon IL-2 challenge will be important for understanding the difference between JAK2- and JAK1- and/or JAK3-mediated activation of STAT5.

It is noteworthy that although both IL-2 and IL-4 activate JAK1 and JAK3 (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994), these cytokines utilize different STATs (Figure 2). Thus the JAK kinase alone cannot be responsible for the selection of STATs to be activated. Receptors may also participate in that choice. In fact, each cytokine stimulates a different subset of STATs via their receptors (for reviews, see Darnell *et al.*, 1994; Ihle *et al.*, 1994). The binding of the cytokine to its receptor often results in tyrosine phosphorylation of the receptor as well as of the intracellular proteins (for reviews, see Miyajima *et al.*, 1992, 1993), suggesting that tyrosine phosphorylation of the receptor is a prerequisite for the stimulation of subsequent substrates in the signaling cascade. For example, the activation of STAT1 by IFN-γ requires the tyrosine residue at position 440 in the cytoplasmic domain of the IFN-γ receptor α subunit (Gleenlund *et al.*, 1994). Presumably, the tyrosine-phosphorylated receptor recruits STAT1 through its SH2 domain. Likewise, the tyrosine residues at position 578 and 606 in the IL-4R are indispensable for the activation of IL-4 STAT (Hou *et al.*, 1994). In contrast, activation of STAT5 by GM-CSF does not require the receptor tyrosine phosphorylation. Although GM-CSF induces tyrosine phosphorylation of the β subunit of the GM-CSF receptor, GM-CSF can activate STAT5 through the truncated GM-CSF receptor β subunit devoid of any tyrosine residues (Mui *et al.*, 1995). Recently we obtained similar results with EPOR (H.W. and G.Krystal, manuscript in preparation). Thus, the specific mechanism of STAT activation still remains to be elucidated.

Activation of JAKs and STATs generally occurs rapidly and transiently. JAK2 phosphorylation by EPO reached a maximum level within 5 min and completely disappeared after 10 min (Witthuhn *et al.*, 1993). The stimulation of STATs lasts for 30 min or longer depending on the STATs (Kotanides and Reich; 1993; Silvennoinen *et al.*, 1993; Akira *et al.*, 1994). In contrast, the activation of JAK2 and STAT5 in TF-1 was sustained for at least 3 h after EPO stimulation (Figure 3B), and was observed even after 24 h (H.W., unpublished results). These data strongly implicate additional factors regulating JAK–STAT activation in TF-1 cells, such as positive or negative regulators of kinases and phosphatases.

### *To respond or not to respond to EPO*

Responsiveness to EPO in CTLL-2 transfectants expressing EPOR has been a controversial subject among various laboratories (Showers *et al.*, 1992; Yamamura *et al.*, 1992,1994; Sakamaki *et al.*, 1993; Barber and D'Andrea 1994). We have presented a unique system for approaching this problem using EPO non-responsive and responsive cells. As the frequency of appearance of EPO-responsive cells from ERT was higher than $10^{-4}$, the conversion to EPO responsiveness is not likely to be due to somatic mutation, but probably to selection of ERT cells capable of responding to EPO. There was no significant difference in the number of EPOR and the affinity of EPO for EPOR or in the expression level of JAK2 protein between ERT and ERT/E2 cells. Nevertheless, EPO induced tyrosine phosphorylation of STAT5 and JAK2 as well as other cellular proteins only in ERT/E2 cells but not in ERT cells [Figure 4A, D and E(1)]. These data implied that expression of EPOR and JAK2 is not sufficient for EPO responsiveness. We found the association of EPOR with JAK2 in ERT/E2, but not in ERT cells (Figure 5), indicating that ERT cells may not express a component(s) required for the association between EPOR and JAK2. Acquisition of this component would impart the EPO competence to ERT/E2 cells. Alternatively, an inhibitory molecule(s) in ERT cells might hamper that interaction. The disappearance or displacement of this molecule could allow JAK2 and EPOR to associate, and thus enables cells to respond to EPO. The JAK2–EPOR interaction has been demonstrated not only in hematopoietic cells but also in non-hematopoietic cells including insect cells (Witthuhn *et al.*, 1993; Miura *et al.*, 1994a; Quelle *et al.*, 1994). These results favor the latter model where an inhibitory molecule(s) present in ERT cells prevents the EPOR–JAK2 association. Our current data collectively demonstrate that there is a difference in EPOR–JAK2 interaction between ERT and ERT/E2 cells, and that these cells may provide a unique system to address EPO responsiveness in T cells.

## Materials and methods

### *Cells*

CTLL-2 cells were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS), 50 μM β-mercaptoethanol and 1 ng/ml *Escherichia coli*-derived mouse IL-2. For nuclear extract preparation and immunoprecipitation, cells were starved for 6 h without IL-2 prior to stimulation. TF-1 cells were maintained in RPMI 1640 medium as above in the presence of human GM-CSF (5 ng/ml). For EPO challenge, cells were starved for 24 h without GM-CSF. ERT and ERT/E2 cells were cultured in the same medium as that of CTLL-2, but in the presence of 1 ng/ml *E.coli* derived mouse IL-2 or 2 U/ml human recombinant EPO (R&D), respectively.

### *Antibodies against STAT5*

N1 and N5 antibodies were raised against the N-terminal (comprising amino acids 6–129) and C-terminal (including amino acids 589–734) region of ovine STAT5. These portions were expressed as GST fusion proteins with pGEX2T vector. Resulting fusion proteins were injected into chickens twice (400 μg protein at each injection) at 3-week intervals. Yolk Ig was prepared by polyethylene glycol precipitation methods.

### *Electrophoretic mobility shift analysis (EMSA)*

Nuclear extracts were prepared with NP-40 (Sadowski and Gilman, 1993), and mixed with the radiolabeled double-stranded oligonucleotide corresponding to the PRE in the bovine β-casein promoter (Wakao *et al.*, 1994). EMSA was performed using 8 μg of nuclear extracts in 20 μl of reaction mixture containing 12 mM HEPES (pH 7.9), 10 fmol of radiolabled PRE (40 000 c.p.m.), 5% glycerol, 75 mM NaCl, 0.1% NP-40, 1 mg/ml BSA, 1 mM EDTA and 1 μg of poly d(I,C). The mixture was incubated at room temperature for 30 min and 5 μl were loaded onto 5% polyacrylamide gel (0.25× TBE). For supershift experiments, 10 μg of pre-immune serum or N1, N5 antiserum were added to the binding reaction.

### *Cell lysis and immunoprecipitation*

ERT and ERT/E2 cells were washed twice with RPMI 1640 medium containing 10% FCS and 50 μM β-mercaptoethanol (starvation medium), suspended in starvation medium at $2\times10^6$ cells/ml and incubated for 6 h at 37°C in the absence of IL-2 or EPO. Cells were then stimulated with mouse IL-2 (10 ng/ml) or EPO (5 U/ml) for 20 min at 37°C, followed by centrifugation at 5000 r.p.m. for 1.5 min in a microcentrifuge. Cells were lysed for 60 min on ice by adding 1 ml of lysis buffer containing 50 mM HEPES buffer (pH 7.4), 1% Triton X-100, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mM Pefablock (Boehringer Mannheim) and 10 μM pepstatin A. Cell lysates were cleared by centrifugation for 15 min at 15 000 r.p.m. and used for the immunoprecipitation. The proteins were immunoprecipitated from cell lysates with anti-JAK1–3 or Tyk2 antibody (UBI) according to the manufacturer's protocol and separated by SDS–PAGE. Proteins were then probed with the monoclonal anti-phosphotyrosine antibody (4G10, UBI) and with the appropriate second antibody and visualized by the ECL system (Amersham).

### *Detection of JAK2 and EPOR association in vivo*

ERT and ERT/E2 cells were starved for 6 h without IL-2 or EPO, then cells were lysed with buffer containing 1% digitonin after 20 min with or without EPO challenge (Miura *et al.*, 1994a) and immunoprecipitated with anti-murine EPOR antibody (provided by Dr A.Yoshimura, Kagoshima University, Japan). Precipitated proteins were separated by SDS–PAGE and probed with 4G10 and anti-JAK2 antibody.

### *Stable transfection of STAT5A and B into ERT/E2 cells*

STAT5A and STAT5B cDNA were cloned into the pME18S vector possessing an influenza tag sequence. A hygromycin resistance gene was then inserted into these constructs. After linearization, STAT5s containing pME18S vectors were individually transfected into ERT/E2 cells by electroporation (960 μF, 400 V) and selected in the presence of hygromycin. Hygromycin-resistant clones were isolated and used in EMSA experiments.

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