Mitogen-activated Protein Kinase Plays an Essential Role in the Erythropoietin-dependent Proliferation of CTLL-2 Cells*

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Erythropoietin (EPO) and its receptor (EPOR) are required for development of erythrocytes. It has been shown that the ectopic expression of EPOR confers EPO-dependent proliferation on an interleukin 3 (IL3)-dependent cell line, Ba/F3, whereas the IL2-dependent T cell line, CTLL-2 expressing the EPOR (T-ER), fails to proliferate in response to EPO. However, the molecular basis of the EPO unresponsiveness in CTLL-2 has not been clarified. We found that the expression level of JAK2 in T-ER cells was much lower than that in Ba/F3 cells. Therefore, we examined the effects of forced expression of JAK2 in T-ER cells. In T-ER transformants expressing JAK2 (T-JER), EPO induced tyrosine phosphorylation of the EPOR, JAK2, and STAT5, and consequently STAT5-responsive genes including bel-X and cisl were normally induced. Furthermore, T-JER cells were resistant to apoptosis until at least 72 h after switching from IL2 to EPO. Although T-JER cells could not continuously proliferate in the presence of EPO, additional expression of JAK2 in T-JER (T-JJER) to a level similar to that in Ba/F3 cells supported long term proliferation in response to EPO. JAK2 was equally co-immunoprecipitated with the EPOR among T-JER, T-JJER, and Ba/F3 cells expressing the EPOR (BF-ER). However, EPO-dependent mitogen-activated protein (MAP) kinase activation was observed in T-JJER and BF-ER cells but not in T-JER cells. EPO-dependent long term proliferation of T-JER cells was conferred by expression of the constitutively activated form of MEK1. Our results suggest that MAP kinase activation is, at least in part, an important component for mitotic signal from the EPOR, and CTLL-2 cells probably lack signaling molecule(s) in JAK2 and the Ras-MAP kinase pathway.

Erythropoietin (EPO) is an essential cytokine for development of committed erythroid progenitor cells (1). EPO binds to and dimerizes the EPO receptor (EPOR), a member of the cytokine superfamily, leading to the activation of JAK2, which is constitutively bound to the EPOR. Activated JAK2 then phosphorylates tyrosine residues of the receptor, which recruits various signaling proteins to the receptor complex (2–5). Among these, STAT5 has been shown to play an important role in expression of the anti-apoptotic molecule Bcl-X (6, 7) and in cytokine-dependent growth (8, 9). On the other hand, interleukin 2 (IL2) is a critical cytokine for T cell proliferation, which activates JAK1 and JAK3 (10–14). However, IL2 also activates STAT5 similarly to EPO or IL3 (15).

The IL3-dependent cell line Ba/F3 has been widely used to investigate proliferation signals from the cytokine receptors, including EPOR (16, 17), granulocyte-colony-stimulating factor receptor (18), gp130 (19), IL3/IL5/granulocyte-macrophage-colony-stimulating factor common β (20), and IL2 receptor β and γ chains (21, 22). Similarly, EPO expression in other IL3-dependent cell lines including 32D (23) and FDC-P1 (24) conferred EPO-dependent growth. In contrast, IL2-dependent T cell lines CTLL-2 provided controversial results concerning proliferation in response to EPO (25–28). One report indicated that EPO expression conferred EPO-dependent growth on a CTLL-2 line, whereas others did not. A previous study indicated that EPO did not induce phosphorylation of the EPOR, although infection with Kirsten murine sarcoma virus (v-Ki-Ras) conferred EPO responsiveness on a CTLL-2 subline (28). The same group also reported that the expression of v-Ki-Ras resulted in co-immunoprecipitation of tyrosine phosphorylated 160-kDa and 130-kDa proteins with the EPOR, although JAK2 was not present in this immune complex. In another study, interaction between JAK2 and EPOR was not detected in EPO-unresponsive CTLL-2 cells expressing the EPOR (5). Recently, Gaffen et al. (29) also found that another IL2-dependent T cell line, HT-2, failed to respond to EPO when the EPOR was expressed. In this case, EPO induced phosphorylation of JAK2 and the EPOR but not STAT5. Complementation experiments using a fusion between HT-2 and Ba/F3 cells indicated that factor(s) essential for signaling from the EPOR are missing in HT-2 cells.

In the present study, we obtained two CTLL-2 cell lines, one that was able to proliferate in response to EPO after ectopic expression of the EPOR, whereas the other was not, and analyzed signaling pathways from the EPOR. We concluded that the low level of JAK2 as well as the lack of a link between JAK2 and MAP kinase pathway are the primary reasons for the EPO unresponsiveness of CTLL-2 cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—We obtained two mouse IL2-dependent cytotoxic T cell lines derived from CTLL-2; one was provided by Dr. Sugamura (Tohoku University) and abbreviated as T-CTLL-2, whereas the other, abbreviated D-CTLL-2, was from Dr. Mui, DNAX Research Institute. Both cell lines were maintained in RPMI 1640 medium con-

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...taining 10% fetal calf serum (FCS) and 10% conditioned medium from P3UI (BCMGS-mIL2) cells as a source of IL2. 10% conditioned medium corresponded to approximately 100 units/ml IL2. Expression of the EPO conferred EPO-dependent long-term proliferation on D-CTLL-2 cells, whereas T-CTLL-2 expressing the EPOR did not proliferate in response to EPO. Mouse IL3-dependent Ba/F3 cells were cultured in RPMI 1640, 10% fetal calf serum, and 10% WEHI conditioned medium as a source of IL3.

Regents and Antibodies—Recombinant human EPO was kindly provided by Kirin Brewery Co. Ltd. (Tokyo, Japan). Anti-JAK2 antibodies were purchased from UBII (062-255) or raised by immunizing a rabbit with purified recombinant JH1 domain of JAK2 fused to glutathione S-transferase. Anti-STAT5 antibody was purchased from Santa Cruz Biotechnology (C-17), and anti-phosphorylated STAT5-specific antibody was from UBII (06-798). Anti-ERK2 antibody was from Santa Cruz Biotechnology (C-14), and anti-active ERK1/2 antibody was from Proligo (S03A). Anti-EPOR antibody was described previously (17).

Plasmid Construction and DNA Transfection—Mouse EPOR in pEF-neo and mouse JAK2 in pEP-BOS were transfected into cells by electroporation as described (30). JAK2 cDNA was subcloned into the expression vector pME-hygro (JAK2/pME-hygro) containing the hygromycin resistance marker. Ba/F3, D-CTLL-2, and T-CTLL-2 transfectants expressing EPO were designated as BF-ER, D-ER, and T-ER, respectively. T-CTLL-2 transformant expressing both EPO and JAK2 was designated as T-JER. T-JER further transformed with JAK2/pME-hygro was designated as T-JER2.

Cell Proliferation Assay—Cell proliferation was measured by a colorimetric assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-dissulphophenyl)-2H-tetrazolium (WST-1, Dojindo, Japan). Exponentially growing cells (2×10^5) were plated on microtiter plates in 100 μl of culture medium in the presence of various concentrations of EPO. After incubation at 37 °C for 4 days, 10 μl of 3.2 mg/ml WST-1 was added to each well and incubated at 37 °C for an additional 1 h. Optical densities were measured using a microplate reader with a test wavelength of 405 nm and a reference wavelength of 620 nm.

Immunoprecipitation and Immunoblotting—After stimulation with 10 units/ml EPO or 10 ng/ml IL2, cells were lysed in lysis buffer (20 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 μM sodium vanadate, 1 mM dithiothreitol, 5 mg/ml leupeptin, and 1 μM phenylmethylsulfonyl fluoride) and centrifuged at 15,000 × g for 15 min. To detect binding between JAK2 and the EPOR, digitonin was used instead of Triton X-100 as a detergent (4). Supernatants were immunoprecipitated with antibodies against the EPOR or JAK2 and then incubated with protein A-Sepharose beads for 2 h at room temperature. The membrane was washed with TBST three times and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 1 h. After washing with TBST three times, blots were visualized by enhanced chemiluminescence (Pierce).

Northern Blotting—Total RNA was separated on 1.0% agarose gels containing 2.4% formaldehyde and then transferred onto positively charged nylon membranes (Hybond N+, Amersham Pharmacia Biotech). After fixation under calibrated UV irradiation, the membranes were hybridized with digitonin-labeled riboprobes prepared using a digitonin-RNA labeling kit (Roche Molecular Biochemicals). The blots were stained using alkaline phosphatase-labeled anti-digitonin antibody and chemiluminescent substrate according to the manufacturer’s instructions. To make digitonin-riboprobe for JAK2, the JAK2 cDNA was digested with EcoRI, and then the EcoRI fragment (1–789) was subcloned into BKS+ –. Probes for bcl-X, cIκB, and c-myc were described previously (31).

Flow Cytometric Analysis—Aliquots of exponentially growing cells (1×10^5) were cultured in the presence or absence of EPO or IL2 for the indicated periods. Then the cell cycle was analyzed with a fluorescence-activated cell sorter (FACS) after staining with propidium iodide (PI) as described previously (32).

RESULTS

Comparison of the Expression of JAK2 in CTL-2 Sublines—There have been conflicting reports concerning EPO responsiveness of CTL-2 cells expressing the EPOR. One report indicated that EPO expression conferred EPO-dependent growth on CTL-2 cells, whereas others did not (25–28). To clarify the reason for this discrepancy and the molecular mechanism of the EPO unresponsiveness in CTL-2 cells, we obtained two CTL-2 sublines (T-CTLL-2 and D-CTLL-2) from different institutes and introduced EPO cDNA into both cell lines. D-CTLL-2 cells expressing EPO (D-ER) were able to proliferate in EPO as fast as IL3-dependent Ba/F3 cells expressing the EPO (BF-ER). However, T-CTLL-2 cell line expressing EPO (T-ER) did not proliferate in response to EPO. EPO did not inhibit apoptosis of T-ER cells after switching from IL2 (Fig. 2, T-ER). First we compared JAK2 expression levels among these cell lines. Northern as well as Western blotting experiments indicated that both JAK2 mRNA and protein levels in D-CTLL-2 and D-ER cells were close to those in Ba/F3 and BF-ER cells, whereas T-CTLL-2 and T-ER cells expressed much lower levels of JAK2 (Fig. 1). These results suggested that one simple explanation for the EPO unresponsiveness in T-ER cells is the low level of expression of JAK2.

Elevated Expression of JAK2 Conferred an EPO-dependent Anti-apoptotic Effect and Gene Expression on T-ER Cells—To examine the possibility that low JAK2 level caused EPO unresponsiveness in T-ER cells, we introduced JAK2 and the EPOR simultaneously into T-CTLL-2 cells. The resulting cells (T-JER) were resistant to apoptosis up to at least 72 h after switching from IL2 to EPO, whereas EPO failed to prevent cell death in both T-ER cells and in T-CTLL-2 cells (Fig. 2A). However T-JER cells did not continuously proliferate in EPO (see Fig. 3A). To compare signals between the EPO and IL2 receptors, we examined gene expression. As shown in Fig. 2B, EPO as well as IL2 induced c-myc, bcl-X, and cIκB in T-JER cells,
although EPO-induced c-myc and bcl-X levels were slightly lower than those induced by IL2. Induction of bcl-X may explain the anti-apoptotic activity of EPO in T-JER cells (Fig. 2B). Because cis1 and bcl-X have been shown to be induced by STAT5, these observations suggested that STAT5 was normally activated by EPO in T-JER cells. Indeed, tyrosine phosphorylation of STAT5 was normally induced in response to EPO in T-JER cells (see Fig. 4A). We also compared gene expression between T-JER and BF-ER cells (Fig. 2C). Although cis1 was induced in T-JER cells at a level similar to that in BF-ER cells, the levels of c-myc and bcl-X in T-JER cells were lower than those in BF-ER cells, suggesting that full induction of these genes may be necessary for long term proliferation.

EPO-induced JAK2 Tyrosine Phosphorylation and Association with the EPOR—We found that the JAK2 expression level in T-JER cells was still slightly lower than that in BF-ER cells (Fig. 3A). Thus, we additionally introduced JAK2 into T-JER cells using the hygromycin resistance marker for selection. Two clones (T-JJER) were obtained, and they expressed similar levels of JAK2 to BF-ER cells (Fig. 3A). These cells were able to proliferate in response to EPO in a dose-dependent manner (Fig. 3B). Thus, we further compared EPOR signal transduction pathways between T-JER, T-JJER, and Ba/F3 cells.

First, we compared the kinetics of tyrosine phosphorylation.

Fig. 2. Anti-apoptotic response in BF-ER and T-JER cells. A, T-JER cells cultured in IL2 (10 units/ml) were washed and then cultured without (Free) or with EPO (1 unit/ml) for the indicated periods. DNA contents were determined by FACS after PI staining. B and C, T-JER and BF-ER cells were stimulated with EPO (10 units/ml) or IL2 (100 units/ml) for the indicated periods (min). Total RNA was hybridized with the indicated riboprobes.
of JAK2 among T-JER, T-JJER, and BF-ER cells. As shown in Fig. 3A, EPO-induced JAK2 phosphorylation was detected in all three cell lines, although it was much weaker in T-JER cells compared with T-JJER and BF-ER cells. Previously Wakao et al. (5) reported that CTLL-2 expressing EPOR did not proliferate in response to EPO because JAK2 did not associate with the EPOR. To examine this possibility, the EPOR was immunoprecipitated and the immunoprecipitates were blotted with anti-JAK2 (Fig. 3D). The amount of JAK2 associated with the EPOR was similar among the three cell lines. The mechanism of lower level activation of JAK2 in T-JER cells is not clear at present. However, these data suggest that EPO induced activation of JAK2 in T-JER cells, but this level of JAK2 activation was not sufficient for long term proliferation.

**Impaired MAP Kinase Activation in T-JER Cells**—To understand the missing pathway in T-JER cells, we analyzed STAT5 and MAP kinase (Erk) activation. STAT5 and the Ras-MAP kinase pathway have been shown to be commonly activated by EPO, IL2, and IL3. As shown in Fig. 4A, STAT5 was similarly phosphorylated in T-JER, T-JJER, and BF-ER cells in response to IL2, IL3, or EPO, although EPO-induced STAT5 phosphorylation was substantially weaker in T-JER cells compared with T-JJER and BF-ER cells. However, EPO induced cis1, a direct target of STAT5 in T-JER at a level comparable to that in BF-ER cells (see Fig. 3B). Thus, STAT5 was sufficiently activated to induce target genes in T-JER cells in response to EPO. Next, we compared the activation of MAP kinase using anti-active MAPK (ERK1/2) specific antibody (Fig. 4B). No differences in ERK1/2 activation between T-JER and T-JJER in response to IL2 were detected, whereas a robust ERK1/2 activation was detected when T-JJER were induced with EPO.

To determine the role of MAP kinase activation in proliferation of CTLL-2 cells, we established T-JER transformants (T-JERMEK) expressing active MEK1 cDNA. T-JERMEK cells exhibited constitutive ERK1/2 activation as expected (data not shown). By expressing active MEK1, T-JER cells acquired the ability to proliferate continuously in response to EPO (Fig. 5). Moreover, we found that some T-JERMEK clones could survive without cytokines for 4 days. These observations indicated that MAPK activation was one of the essential growth signals induced by the EPOR, and efficient activation of this pathway may not occur in T-CTLL and T-JER cells.

**DISCUSSION**

Although growth of hematopoietic cells is controlled by a variety of cytokines, the precise mechanisms of signal transduction from cytokine receptors for growth and anti-apoptotic effects have not been elucidated. Ectopic expression of EPOR has been shown to confer EPO-dependent proliferation on Ba/F3 cells but not on certain CTLL-2 sublines. This system could provide important insights into the signaling pathways from the EPOR that are essential for proliferation.

In vertebrates, four members of the JAK family of tyrosine kinase have been identified, and each member has been shown
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To play a central role in the function of the cytokine receptors. Fetal liver cells from JAK2-deficient mice failed to respond to EPO but were rescued by infection with a retrovirus carrying JAK2 cDNA (33, 34). These results demonstrated that JAK2 is essential for EPOR function. Previous studies concluded that there were no differences in the expression level of JAK2 protein between two CTLL-2 cell lines (5). However, when BF-ER cells were cultured in 10% WEHI conditioned medium or T-JER and T-JERMEK clones in 100 units/ml IL2.

different levels of JAK2 (T-JER and T-JJER). Since elevated expression of JAK2 in T-CTLL cells expressing the EPOR (T-JJER) conferred EPO-dependent long term proliferation, we conclude that one of the primary reasons for the EPO unresponsiveness of T-CTLL cells was a low level of JAK2 expression.

Wakao et al. (5) reported that tyrosine phosphorylation of cellular proteins was not induced in an EPO-unresponsive CTLL-2 subline expressing the EPOR, and they found that JAK2 was not associated with the EPOR in ERT cells (5). Similarly, Yamamura et al. (28) reported that JAK2 was not present in the EPOR complex from a CTLL-2 subline (C/Eras4), which expressed both v-Ki-Ras and EPOR (28). Our results shown in Fig. 3 were not consistent with theirs. We demonstrated that EPO unresponsiveness in CTLL-2 expressing the EPOR cannot be explained by uncoupling between JAK2 and the EPOR. This discrepancy might have been due to differences in conditions used for solubilization of the EPOR complex or due to differences in the CTLL-2 cell lines. We also observed that JAK2 level in T-JER cells may not reach a critical threshold concentration that is sufficient to produce long term proliferation signals because T-JJER cells that express higher levels of JAK2 grew continuously in EPO.

Recently, it has been demonstrated that cytokines exert both anti-apoptotic and cell cycle-driving signals. Gaffen et al. (29) reported that the EPOR transmitted anti-apoptotic signals but not proliferation signals in IL2-dependent HT cells. They could not detect the activation of STAT5 despite phosphorylation of the EPOR and JAK2, and they suggested that the anti-apoptotic signal of the EPOR in HT-2 cells is different from Bcl-2, Bcl-X, and MAP kinase activation. We also observed that EPO

Fig. 4. EPO-induced STAT5 and ERK activation in CTLL-2 or Ba/F3 transformants. T-JER, T-JJER, and BF-ER cells were treated with EPO (10 units/ml) or IL2 (100 units/ml) or 1% WEHI conditioned medium (IL3) for the indicated periods (min). The cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted with anti-tyrosine phosphorylated STAT5 (A, α-PY-STAT5) or anti-phosphorylated ERK1/2 (B, α-P-ERK1/2) antibodies. Subsequently, the membranes were reprobed with anti-STAT5 (A, α-STAT5) or anti-ERK2 (B, α-ERK2).

Fig. 5. Effects of active MEK1 expression in T-JER cells on EPO-dependent proliferation. T-JER cells transformed with constitutively activated MEK1 (T-JERMEK) and parental T-JER cells were cultured in the presence of the indicated concentrations of EPO. After incubation for 4 days, proliferation of cells was measured by the WST-1 colorimetric assay. Control cell proliferation value (100%) was obtained when BF-ER cells were cultured in 10% WEHI conditioned medium or T-JER and T-JERMEK clones in 100 units/ml IL2.
could exert anti-apoptotic effects in T-JER cells, probably through the activation of STAT5. Consistent with this hypothesis, bel-X mRNA was induced in T-JER stimulated with EPO (Fig. 3B). It is generally accepted that Bel-X is a critical factor for survival of erythroid progenitor cells as well as many cytokine-dependent hematopoietic cell lines (35). It has been shown that STAT5, at least in part, plays an important role in Bel-X induction (6, 7, 36, 37). Our results supported this hypothesis, although the anti-apoptotic effect in T-JER cells might depend on a pathway different from that in HT-2 cells. Further studies are necessary to clarify the differences between T-JER and HT cells.

EPO activated MAP kinase in T-JER but not in T-JER cells, suggesting that MAP kinase activation is an important component of the proliferative signal from the EPO. Although it has been controversial whether MAP kinase cascades are necessary for proliferation by IL3 and EPO (8, 9, 38–40), Kinoshita et al. (39) reported that infection of CTLL-2 cells with v-Ki-Ras resulted in proliferation of CTLL-2 cells. We also observed that T-JERMEK cells, which process IL6/gp130 system (42), we also observed that T-JERMEK cells, which process IL6/gp130 system (42), been controversial whether MAP kinase cascades are necessary to clarify the differences between T-JER and HT cells. Further studies are necessary to clarify the differences between T-JER and HT cells.

These observations raise the question of what are the factors lacking between Jak2 and RAS-MAP kinase in T-CTL2. Such factors presumably exist in Ba/F3 cells. Yamamura et al. (41) reported that Jak2 is necessary for the anti-apoptotic effect in Ba/F3 cells (41). The Ras-MAP kinase pathway has been shown to be necessary for cell cycle progression through the IL6/gp130 system (42). We also observed that T-JERMEK cells, T-JER transformants expressing active-MEK1, conferred MAP kinase phosphorylation and proliferation in response to EPO. Moreover, MAP kinase phosphorylation was observed in all CTLL-2 transformants in response to IL2. Thus, MAP kinase can be activated efficiently through the IL2 receptor/JAK1, JAK3 system but not through the EPO/JAK2 system in T-CTL2 cells.

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