Erythropoietin and Interleukin-2 Activate Distinct JAK Kinase Family Members

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The erythropoietin (EPO) receptor and the interleukin-2 (IL-2) receptor β -chain subunit are members of the cytokine receptor superfamily. They have conserved primary amino acid sequences in their cytoplasmic domains and activate phosphorylation of common substrates, suggesting common biochemical signaling mechanisms. We have generated a cell line, CTLL-EPO-R, that contains functional cell surface receptors for both EPO and IL-2. CTLL-EPO-R cells demonstrated similar growth kinetics in EPO and IL-2. Stimulation with EPO resulted in the rapid, dose-dependent tyrosine phosphorylation of JAK2. In contrast, stimulation with IL-2 or the related cytokine IL-4 resulted in the rapid, dose-dependent tyrosine phosphorylation of JAK1 and an additional 116-kDa protein. This 116-kDa protein was itself immunoreactive with a polyclonal antiserum raised against JAK2 and appears to be a novel member of the JAK kinase family. Immune complex kinase assays confirmed that IL-2 and IL-4 activated JAK1 and EPO activated JAK2. These results demonstrate that multiple biochemical pathways are capable of conferring a mitogenic signal in CTLL-EPO-R cells and that the EPO and IL-2 receptors interact with distinct JAK kinase family members within the same cellular background.

The erythropoietin (EPO) receptor (EPO-R) and the interleukin-2 (IL-2) receptor β -chain subunit (IL-2-R β) are related members of the cytokine receptor superfamily and share several extracytoplasmic structural features. In addition, the EPO-R and IL-2-R β have 45% amino acid identity within the box 1 and box 2 regions of their cytoplasmic regions (9, 14). However, the remaining cytoplasmic regions of the IL-2-R β and EPO-R are highly divergent. The EPO-R is a 507-amino-acid polypeptide that forms homodimers following EPO stimulation (61). The existence of multiple cross-linked complexes (33, 34, 40, 51) and multiple affinities (50, 51) suggests the possibility of additional EPO-R subunits. The IL-2-R functions as a multisubunit structure composed of α , β , and γ chains (45). The IL-2-R γ is a common subunit (IL-2-R γ_c) shared with the IL-4 (29, 48) and the IL-7 (28, 44) receptors.

Previous studies have suggested that the EPO-R and the IL-2-R activate distinct downstream signals (65), although no studies have investigated the biochemical signaling events in cells that express both receptor types. Activation of the EPO-R (64) or the IL-2-R β (15) results in the induction of tyrosine kinase activity and tyrosine phosphorylation of several proteins. Although neither receptor has intrinsic tyrosine kinase activity, activation of cytoplasmic tyrosine kinases correlates with mitogenesis for these receptors.

Activation of the EPO-R results in the rapid tyrosine phosphorylation of the EPO-R itself (5, 8, 11, 39) as well as the cytoplasmic tyrosine kinase JAK2 (5, 64). The tyrosine kinase Fes has also been shown to be tyrosine phosphorylated in response to EPO in TF-1 cells, a human erythroleukemia cell line (18). However, association of Fes with the EPO-R was not tested, and Fes activation was not observed in DA-3-EPO-R cell line (64). EPO-R activation may activate a number of

intracellular tyrosine kinases such as shown for the T-cell receptor (42, 62).

Activation of the IL-2-R results in tyrosine phosphorylation of the IL-2-R β (4, 15, 36, 52), of the IL-2-R γ (2), and of multiple substrates migrating at 180, 116, 97, 70, 55, and 42 kDa (26). The IL-2-R β associates with various Src family kinases such as Lck in CTLL cells (21, 22, 37) or Lyn and Fyn in Ba/F3-BO3-IL-2-R β cells (27). However, the IL-2-R β is tyrosine phosphorylated in cell lines that lack Lck expression, including L cells (3, 30), a cell line of fibroblast origin, suggesting that other cytoplasmic tyrosine kinases may be activated by IL-2. JAK tyrosine kinases are widely expressed in many different tissues and cell lines (12, 20, 63) and are excellent candidates to be involved in the IL-2 signaling pathway. It is not known which, if any, JAK family kinase members are activated by IL-2.

The JAK family of tyrosine kinases plays a role in the signal transduction pathway of cytokine receptors. JAK2 was first identified by PCR amplification of conserved DNA sequences encoding tyrosine kinases domains in hematopoietic cells (20). JAK2 belongs to the Janus family of cytoplasmic tyrosine kinases, which also includes JAK1 (63) and Tyk2 (12). The members of this family are characterized by the presence of a second kinase-like domain and the absence of SH2 and SH3 domains. Stimulation of the DA-3-EPO-R cell line with EPO activated JAK2 tyrosine phosphorylation and in vitro kinase activity (64). In addition, JAK1, JAK2, and Tyk2 have been implicated in the signal transduction pathways of multiple cytokine receptors (1, 32, 41, 43, 54, 55, 57, 60). The recent observation that the IL-2-R γ_c interacts with the receptors for IL-4 (29, 48) and IL-7 (28, 44) also suggests that these receptor complexes may recruit a similar subset of JAK tyrosine kinases.

Considering that tyrosine kinase domains have regions of sequence similarity (19), we sought to develop an antibody against JAK2 which also might cross-react with other potentially novel JAK kinases. We generated a polyclonal antiserum against the functional, carboxy-terminal kinase domain (JH1) of JAK2 and used this reagent as a biochemical tool to

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investigate activation of JAK kinases in various cell lines. For this purpose, we examined a CTLL cell line ectopically expressing the EPO-R, hereafter referred to as CTLL-EPO-R (53). This allowed us to examine the activation of multiple JAK tyrosine kinases by IL-2, IL-4, or EPO, using a cross-reactive JAK antibody (R80). Our results demonstrate that EPO and IL-2 activate different early biochemical signaling responses, although mitogenesis can be achieved through either pathway.

MATERIALS AND METHODS

Cells and cell culture. HT-2 (25) and CTLL-2 cells, as previously described (53), were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (FCS) and 2 U of human recombinant IL-2 (Boehringer-Mannheim) per ml (IL-2 medium). YT cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) FCS (66).

DNA transfection of CTLL cells. CTLL cells (10^7) were transfected by coelectroporation with a PXM-EPO-R cDNA (linearized with *NdeI*) and with PSV2neo (linearized with *AccI*) as previously described (53). Selection with G418 (1.0 mg/ml) in IL-2 medium was initiated 48 h after electroporation. Selected cells were subcloned by limiting dilution in G418-IL-2 medium.

Flow cytometric analysis. CTLL subclones expressing the EPO-R were washed in RPMI 1640–10% FCS without supplemented EPO or IL-2 and resuspended in phosphate-buffered saline (PBS). The cells (10^6) were incubated with a single, saturating concentration of a protein A-purified polyclonal antiserum (10 mg/ml) directed against the extracytoplasmic domain of the human EPO-R (5) in a 200- μ l reaction mixture. The cells were washed and stained with fluorescein isothiocyanate-conjugated anti-mouse second antibody. Cells were analyzed by FACScan (Becton Dickinson, San Jose, Calif.).

EPO-dependent growth characteristics of CTLL subclones. Individual subclones expressing the EPO-R were assayed for growth in RPMI 1640 medium supplemented with 10% FCS and several concentrations of recombinant human EPO. Cell growth and viability were measured by using the MTT (dimethylthiazol diphenyltetrazolium bromide) assay as previously described (67). Electroporations were performed three times. When resulting transfectants were isolated and tested for EPO responsiveness, identical results were obtained each time.

Metabolic labeling and immunoprecipitation. CTLL subclones were metabolically labeled with [35 S]methionine and [35 S]cysteine as previously described (5). Labeled proteins were immunoprecipitated with antiserum against the amino terminus of murine EPO-R.

Production of antibody R80. A cDNA encoding the carboxy-terminal kinase domain of JAK2 (nucleotides 2544 to 3387) (generously provided by James Ihle and Bruce Witthuhn) was amplified by PCR and subcloned into T vector (Invitrogen). The cDNA was next subcloned, using *Bam*HI and *Eco*RI sites, into pGEX-2T. Glutathione *S*-transferase–JAK2 fusion protein (57 kDa) was produced as described previously (13) and used as an antigen at 100 μ g per injection for the generation of rabbit polyclonal antiserum R80.

Analysis of JAK kinase phosphorylation. CTLL-EPO-R subclones were starved in RPMI 1640–10% FCS for an 8-h period and stimulated for various periods with no added factor, IL-2, IL-4 (Genzyme), or EPO. Cell lysates were prepared by using 1% (wt/vol) Triton X-100 or 1% (wt/vol) Brij 96 (5). Immunoprecipitations were conducted with either anti-JAK/Tyk antibody R80, an anti-JAK1 antibody (UBI [Upstate Biotechnology, Inc.] 06-254 or 06-272), anti-JAK2 antibody

(UBI), or an anti-Tyk2 antibody (Santa Cruz Biotechnology). Immune complexes were isolated on protein A-Sepharose, washed three times with 50 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.1% detergent (Triton X-100 or Brij 96), and prepared for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) as described previously (5).

For immunodepletion experiments, an immunoprecipitation was first performed with a peptide-specific antiserum to JAK1, JAK2, or Tyk2. The supernatant was then incubated with R80, and a second immunoprecipitation was completed. Samples were prepared for SDS-PAGE as described above.

After electrophoretic transfer to nitrocellulose, the membrane was blocked, incubated with 1 μ g of antiphosphotyrosine monoclonal antibody 4G10 per ml for 1 h, washed, and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (1:5,000; Amersham, Arlington Heights, Ill.) for 30 min. Following enhanced chemiluminescence immunodetection, the membrane was incubated in 62.5 mM Tris-HCl (pH 6.8)–2% (wt/vol) SDS–100 mM β -mercaptoethanol at 50°C for 1 h. Membranes were stripped, blocked, and reprobed with either an anti-JAK1 (1:1,000), anti-JAK2 (1:1,000), or anti-Tyk2 (1 μ g/ml) antibody and washed. Horseradish peroxidase–protein A (1:5,000) was added for 30 min, and the membranes were washed prior to enhanced chemiluminescence detection.

In vitro kinase assay. Immune complexes were isolated as described above. Samples were washed twice in 50 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.1% Brij 96 and once in kinase buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 50 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 100 μ M Na₂VO₄) as described previously (64). Kinase reactions were performed for 15 min at room temperature (250 μ Ci of [γ - 32 P]ATP per ml). Reactions were terminated by the addition of 10 mM HEPES (pH 7.4)–10 mM EDTA, and the samples were prepared for SDS-PAGE and autoradiography.

RESULTS

EPO- and IL-2-dependent growth characteristics of CTLL-EPO-R cells. The murine cytotoxic T-lymphocyte line CTLL has an absolute dependence on IL-2 for growth (16) and has no endogenous EPO-R. We cotransfected CTLL cells, by electroporation, with PSV2neo and PXM-EPO-R, and two CTLL-EPO-R subclones were isolated by limiting dilution in G418. As shown by fluorescence-activated cell sorting (FACS) analysis (Fig. 1A), the parental CTLL cell line had no cell surface EPO-R, but the two CTLL-EPO-R subclones expressed cell surface EPO-R. Immunoprecipitation of 35 S-labeled proteins demonstrated that both CTLL-EPO-R subclones expressed wild-type (66-kDa) EPO-R (Fig. 1B, lanes 2 and 3).

We next tested CTLL subclones for IL-2- and EPO-dependent growth (Fig. 2). Half-maximal growth of CTLL-EPO-R was observed at 50 pM IL-2 or 50 mU of EPO per ml. Similar results were obtained with five independently isolated subclones. Furthermore, all five subclones were capable of EPO-dependent proliferation for extended periods of time (data not shown). Subclones of the CTLL parental line grew only in IL-2 and were not EPO responsive. In addition, all CTLL and CTLL-EPO-R subclones grew in the presence of murine IL-4 (data not shown), consistent with previous studies demonstrating that the CTLL cell line expresses IL-4-R (6, 23, 49).

EPO and IL-2 activate distinct JAK kinase family members. Since both IL-2 and EPO provided a mitogenic signal to CTLL-EPO-R, we next tested early biochemical signaling events following receptor activation. Previous studies have shown that activation of the EPO-R results in the tyrosine

FIG. 1. Expression of the heterologous EPO-R polypeptide in CTLL cells. (A) Various CTLL subclones were incubated with 5 nM anti-human EPO-R polyclonal antibody (Ab) and stained with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibody. Fluorescence was analyzed by FACSscan analysis. Note the shifts detected in CTLL-EPO-R subclones 5 and 22. (B) Cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine. Immunoprecipitation with an anti-EPO-R N-terminus antiserum was performed. Lanes: 1, CTLL-2; 2, CTLL-EPO-R subclone 5; 3, CTLL-EPO-R subclone 22. The migration of the EPO-R is indicated. Sizes are indicated in kilodaltons.

phosphorylation of JAK2 (5, 64). To test for JAK tyrosine phosphorylation, we depleted CTLL-EPO-R cells of exogenous growth factors and stimulated them with either no growth factor, IL-2, IL-4, or EPO. Members of the JAK kinase family were immunoprecipitated with antibodies to JAK1, JAK2, and Tyk2 and with an antiserum generated in our laboratory. A

glutathione S-transferase fusion protein consisting of the JH1 domain of JAK2 was used as an antigen, and a polyclonal, cross-reactive anti-JAK antibody (R80) was obtained. Tyrosine-phosphorylated proteins were identified by immunoblotting with an antiphosphotyrosine monoclonal antibody, 4G10 (Fig. 3A, pTyr [phosphotyrosine] immunoblot).

FIG. 2. Cytokine-dependent growth characteristics of CTLL-EPO-R subclones. The indicated cells were washed three times and resuspended in RPMI 1640–10% FCS (5×10^4 cells per 0.2 ml) in the presence of various concentrations of either IL-2 (A) or EPO (B). After 2 days, cell viability was assayed by the MTT assay as previously described (67). Cell lines analyzed were CTLL subclone 1 (●), CTLL subclone 2 (○), CTLL-EPO-R subclone 5 (▲), and CTLL-EPO-R subclone 22 (△). OD, optical density.

FIG. 3. Differential activation of JAK kinase family members by IL-2, IL-4, and EPO. (A) CTLL-EPO-R cells (subclone 5) were washed three times in PBS and stimulated with either no growth factor (lanes 1, 5, 9, and 13), murine IL-2 (lanes 2, 6, 10, and 14), murine IL-4 (lanes 3, 7, 11, and 15), or human EPO (lanes 4, 8, 12, and 16) for 5 min. Cells were lysed, and proteins were immunoprecipitated with antibody R80 (lanes 1 to 4), anti-JAK1 (lanes 5 to 8), anti-JAK2 (lanes 9 to 12), or anti-Tyk2 (lanes 13 to 16). Immune complexes were resolved by SDS-PAGE and blotted to nitrocellulose. (B) Total cell lysates used in the immunoprecipitation in panel A. CTLL-EPO-R cells were stimulated with no growth factor (lane 1), IL-2 (lane 2), IL-4 (lane 3), and EPO (lane 4). Seventy-five micrograms of lysate was resolved by SDS-PAGE and transferred to nitrocellulose. The immunoblots were probed with antiphosphotyrosine monoclonal antibody 4G10. The blots were stripped and reprobed with either an anti-JAK1, anti-JAK2, or anti-Tyk2 peptide-specific antibody as indicated. Positions of migration of JAK1, JAK2, and p116 are shown. IP, immunoprecipitation; Ab, antibody. M_r s are indicated in thousands.

EPO activated the tyrosine phosphorylation of JAK2 (Fig. 3A, lanes 4 and 12). In contrast, IL-2 activated the tyrosine phosphorylation of JAK1 (Fig. 3A, lanes 2 and 6) and an additional 116-kDa phosphoprotein (Fig. 3A, lane 2). This 116-kDa protein was immunoprecipitated with antibody R80 (Fig. 3A, lane 2) but was not immunoprecipitated with a peptide-specific antiserum to JAK1 (UBI 06-272) (Fig. 3A, lane 6), to JAK2 (Fig. 3A, lane 10), or to Tyk2 (Fig. 3A, lane 14). This 116-kDa protein is therefore not JAK1, JAK2, or Tyk2 and is a putative novel member of the JAK kinase family. Interestingly, murine IL-4 activated the tyrosine phosphorylation of the same doublet as IL-2 (Fig. 3A, lane 3); the upper band of the doublet was confirmed to be JAK1 (Fig. 3A, lane 7). As observed for IL-2, murine IL-4 did not activate JAK2 (Fig. 3A, lane 11) or Tyk2 (Fig. 3A, lane 15) tyrosine phosphorylation.

To confirm efficient immunoprecipitation of the various JAK kinase polypeptides, the blot was consecutively stripped and reprobed with either an anti-JAK1, anti-JAK2, or anti-Tyk2 peptide-specific antiserum (Fig. 3A). Antiserum R80 immunoprecipitated JAK1, JAK2, and Tyk2 (Fig. 3A, lanes 1 to 4). The anti-JAK1 antiserum immunoprecipitated only JAK1 (JAK1 immunoblot, lanes 5 to 8). The anti-JAK2 antiserum immunoprecipitated JAK2 (JAK2 immunoblot, lanes 9 to 12) and Tyk2 (Tyk2 immunoblot, lanes 9 to 12). The anti-Tyk2 antiserum immunoprecipitated only Tyk2 (Tyk2 immunoblot, lanes 13 to 16). The JAK2 antibody probably directly immunoprecipitates Tyk2, since no JAK2 is found in

Tyk2 immunoprecipitations (JAK2 immunoblot, lanes 13 to 16).

An antiphosphotyrosine immunoblot of total cell lysates from IL-2, IL-4, or EPO stimulation also demonstrate different patterns of phosphorylated proteins (Fig. 3B). IL-2 and IL-4 predominately activate a 116-kDa phosphoprotein (Fig. 3B, lanes 2 and 3). A tyrosine-phosphorylated protein consistent with the mobility of JAK1 is observed with IL-2 stimulation (Fig. 3B, lane 2), and a phosphoprotein likely to be JAK2 is observed after incubation with EPO (Fig. 3B, lane 4). Additionally, a 97-kDa tyrosine-phosphorylated protein is activated by IL-2 (Fig. 3B, lane 2) (31, 36) or EPO (8, 11, 31, 53) (Fig. 3B, lane 4) stimulation, similar to findings in published reports. IL-2 activates the tyrosine phosphorylation of a 56-kDa substrate (Fig. 3B, lane 2), likely to be Shc (7, 47) or Lck (21, 22, 37). Reprobing of this immunoblot with JAK1, JAK2, and Tyk2 peptide-specific antibodies demonstrated the presence of these proteins in total cell lysates (Fig. 3B).

Identification of specific JAK kinase family members activated by IL-2 and EPO. To verify the identities of the various JAK kinases, we performed immunodepletion experiments (Fig. 4). Immunodepletion with a peptide-specific anti-JAK1 antiserum followed by immunoprecipitation with antibody R80 resulted in a partial loss of the JAK1 signal (Fig. 4A, pTyr immunoblot, lane 8 versus lane 2). This result confirms that IL-2 activates the tyrosine phosphorylation of JAK1. Following immunodepletion, the JAK1 signal is decreased on the antiphosphotyrosine immunoblot (Fig. 4A, pTyr immunoblot,

FIG. 4. Identification of specific JAK kinase members activated by IL-2 and EPO. CTLL-EPO-R cells (subclone 5) were washed three times in PBS and stimulated with either no growth factor (lanes 1, 4, and 7), 50 U of murine IL-2 per ml (lanes 2, 5, and 8), or 50 U of human EPO per ml (lanes 3, 6, and 9) for 5 min. (A) Cells were lysed, and proteins were immunoprecipitated with antibody R80 (lanes 1 to

lanes 7 to 9) but is absent on the anti-JAK1 immunoblot (Fig. 4A, JAK1 immunoblot, lanes 7 to 9) because of the higher sensitivity of antiphosphotyrosine monoclonal antibody 4G10.

Immunodepletion with a peptide-specific anti-JAK2 antiserum followed by immunoprecipitation with antibody R80 resulted in loss of the EPO-induced JAK2 signal (Fig. 4B, lane 9 versus lane 3). This result confirms that JAK2 is activated upon EPO stimulation.

Immunodepletion with a peptide-specific anti-Tyk2 antiserum followed by immunoprecipitation with antibody R80 resulted in no loss of either the EPO-induced or IL-2-induced signal (Fig. 4C, lanes 1 to 3 versus lanes 6 to 9). Therefore, neither EPO nor IL-2 activates the tyrosine phosphorylation of Tyk2 in these cells.

p116 is a unique polypeptide activated by IL-2 and IL-4. Several independent results suggest that p116 is a unique protein and not a degradative product of JAK1. First, p116 is observed only in R80, not JAK1, immunoprecipitations (Fig. 4A, pTyr immunoblot; compare lanes 2 and 5). Second, p116 is not present upon immunoblotting with a JAK1 antibody specific for an internal JAK1 epitope (amino acids 785 to 804) (Fig. 4A, JAK1 immunoblot, lanes 1 to 6). A small loss of p116 signal was also observed upon JAK1 immunodepletion (Fig. 4A, pTyr blot; compare lanes 2 and 8). A similar loss of signal was observed, however, when primary immunoprecipitations were performed with R80 preimmune serum followed by R80 secondary immunoprecipitation (data not shown). Therefore, the decrease in signal intensity arose from the nonspecific loss of protein during the first immunoprecipitation step.

To further examine the relationship between JAK1 and p116, an immunoprecipitation was performed with a peptide-specific JAK1 antibody following lysis with the nonionic detergent Brij 96, which has been shown to preserve protein-protein interactions (10). When CTLL-EPO-R cells are lysed in Brij 96, tyrosine-phosphorylated p116 coimmunoprecipitates with JAK1 after stimulation with IL-2 (Fig. 5A, pTyr immunoblot, lane 6). The migration of p116 is consistent with the lower band of the IL-2-stimulated doublet identified in R80 immunoprecipitations (Fig. 5A, pTyr immunoblot, lane 2). However, only JAK1 is specifically immunoprecipitated in Triton X-100 with a JAK1 antibody (Fig. 5A, pTyr immunoblot, lane 4). As in Fig. 4A, JAK1 but not p116 was immunoreactive on immunoblotting with a peptide-specific JAK1 antibody (Fig. 5A, JAK1 immunoblot). There is an association between JAK1 and p116 detected in Brij 96 that is disrupted in Triton X-100.

To further characterize the novel doublet activated by IL-2 or IL-4, two additional cell lines were examined. R80 immunoprecipitations identified that human IL-2 stimulates the tyrosine phosphorylation of a doublet in YT cells, a human natural killer cell-derived cell line (66) (Fig. 5B, lane 2). In addition, murine IL-2 (Fig. 5B, lane 4) or murine IL-4 (Fig. 5B, lane 5) activated the tyrosine phosphorylation of a doublet in

3), anti-JAK1 antibody (lanes 4 to 6), or anti-JAK1 antibody followed by antibody R80 (lanes 7 to 9). (B) Cells were lysed, and proteins were immunoprecipitated with antibody R80 (lanes 1 to 3), anti-JAK2 antibody (lanes 4 to 6), or anti-JAK2 antibody followed by antibody R80 (lanes 7 to 9). (C) Cells were lysed, and proteins were immunoprecipitated with antibody R80 (lanes 1 to 3), anti-Tyk2 antibody (lanes 4 to 6), or anti-Tyk2 antibody followed by antibody R80 (lanes 7 to 9). Immune complexes were resolved by SDS-PAGE and blotted to nitrocellulose. The immunoblot was probed with the antiphosphotyrosine (pTyr) monoclonal antibody 4G10. The blot was stripped and reprobed with anti-JAK1 (A), anti-JAK2 (B), and anti-Tyk2 (C) peptide-specific antibodies. IP, immunoprecipitation; Ab, antibody.

FIG. 5. p116 is a unique polypeptide activated by IL-2 and IL-4. (A) CTLL cells were incubated in the absence of growth factor for 8 h and then stimulated with no added factor (lanes 1, 3, and 5) or 50 U of IL-2 per ml (lanes 2, 4, and 6). Following cell lysis in 1% Triton X-100 (lanes 1 to 4) or 1% Brij 96 (lanes 5 and 6), an immunoprecipitation was conducted with either antibody R80 (lanes 1 and 2) or an anti-JAK1 antibody (lanes 3 to 6). Following SDS-PAGE and transfer to nitrocellulose, the immunoblot was probed with the antiphosphotyrosine (pTyr) monoclonal antibody 4G10. The blot was stripped and reprobed with an anti-JAK1 peptide-specific antibody. IP, immunoprecipitation; Ab, antibody. (B) YT and HT-2 cells were incubated in the absence of growth factor for 4 h and then stimulated with no added factor (lanes 1 and 3), 50 U of human IL-2 per ml (lane 2), 50 U of murine IL-2 per ml (lane 4), or 50 U of murine IL-4 per ml (lane 5). Following cell lysis, an immunoprecipitation was performed with polyclonal antibody R80. Western blotting using the antiphosphotyrosine monoclonal (pTyr) antibody 4G10 was conducted. M_r s of molecular weight standards are indicated in thousands.

HT-2 cells, similar to CTLL-EPO-R cells as shown in Fig. 3 and 4. Other experiments demonstrate that as in CTLL cells, the upper band of the doublet phosphorylated in response to IL-2 and IL-4 in both YT and HT-2 cells corresponds to JAK1 (data not shown). Therefore, in both human and murine cell lines, IL-2 activates JAK1 and an additional 116-kDa protein.

Time- and dose-dependent activation of JAK kinase family members by IL-2 and EPO. We next tested the cytokine dose-dependent activation of JAK kinases (Fig. 6). The IL-2-dependent activation of both JAK1 and p116 was observed throughout the concentration range (2 to 200 U of IL-2 per ml) tested. The levels of tyrosine phosphorylation of JAK1 and p116 were comparable at all concentrations tested. EPO at 2 to 200 U/ml activated tyrosine phosphorylation of JAK2. Maximal phosphorylation of JAK kinases was observed at concentrations exceeding 20 U of IL-2 (26) and 20 U of EPO (64) per ml.

The kinetics of JAK kinase activation in CTLL-EPO-R cells was next examined (Fig. 7). IL-2 activated the rapid tyrosine phosphorylation of JAK1 and p116 within 1 min, and the level peaked at 15 min. Activation of both JAK1 and p116 showed similar kinetics; no temporal differences in tyrosine phosphorylation of these proteins were observed. In addition, there appeared to be no differences in the relative intensities or the presence of a precursor-product relationship between JAK1 and p116, providing further evidence that p116 is a unique protein. The EPO-inducible tyrosine phosphorylation of JAK2 was observed within 1 min and peaked at 15 min.

Activation of JAK1 tyrosine kinase activity by IL-2 and IL-4. To further confirm that IL-2 activates JAK1 and that EPO activates JAK2 in CTLL-EPO-R cells, we next performed

FIG. 6. Dose dependence of JAK kinase activation by IL-2 and EPO in CTLL-EPO-R cells. CTLL-EPO-R cells (subclone 22) were incubated in the absence of growth factor for 8 h and then stimulated with no added factor (lanes 1 and 7) or various concentrations of IL-2 (lanes 2 to 6) or EPO (lanes 8 to 12) for 5 min. Following cell lysis, an immunoprecipitation was conducted with polyclonal antibody R80. Western blotting using the antiphosphotyrosine monoclonal antibody 4G10 was performed. M_r s of molecular weight standards are indicated in thousands.

immune complex kinase assays of immunoprecipitates obtained with a peptide-specific anti-JAK1 or anti-JAK2 antiserum (Fig. 8). When JAK1 immune complexes from IL-2-stimulated cells were subjected to an in vitro kinase reaction, a 122-kDa protein was tyrosine phosphorylated. Also, IL-4 stimulation, to a lesser extent, led to an increase in JAK1 tyrosine kinase activity. In contrast, a phosphorylated protein of 120 kDa was observed when JAK2 immune complexes from EPO-

FIG. 7. Time course of JAK kinase activation by IL-2 and EPO in CTLL-EPO-R cells. CTLL-EPO-R cells (subclone 22) were incubated in the absence of growth factor for 8 h and then stimulated with no added factor (lanes 1 and 7), 50 U of IL-2 per ml (lanes 2 to 6), or 50 U of EPO per ml (lanes 8 to 12) for the times shown. Following cell lysis, an immunoprecipitation was conducted with polyclonal antibody R80. Western blotting using the antiphosphotyrosine monoclonal antibody 4G10 was performed. M_r s of molecular weight standards are indicated in thousands.

FIG. 8. Activation of *in vitro* kinase activity by IL-2 and EPO. After an 8-h starvation, CTLL-EPO-R cells (subclone 5) were stimulated with no growth factor, 50 U of IL-2 per ml, 50 U of IL-4 per ml, or 50 U of EPO per ml for 10 min. Cells were lysed, and equivalent amounts of cell lysates were subjected to immunoprecipitation with anti-JAK1 or anti-JAK2. *In vitro* kinase assays were performed as described in Materials and Methods. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. The positions of tyrosine phosphorylated JAK1 and JAK2 are indicated by arrows. M_r s are indicated in thousands.

stimulated cells were subjected to an *in vitro* kinase reaction. R80 did not immunoprecipitate *in vitro* kinase activity (data not shown); this antibody may be inhibitory, as the antigen corresponds to the functional kinase domain (JH1) of JAK2. Taken together, these results strongly suggest that IL-2 and IL-4 activate the tyrosine phosphorylation and *in vitro* kinase activity of JAK1, whereas EPO activates JAK2.

DISCUSSION

This study demonstrates that the EPO-R and the IL-2-R β , despite limited regions of cytoplasmic amino acid identity, couple to distinct JAK tyrosine kinase pathways in CTLL-EPO-R cells. EPO activates the tyrosine phosphorylation and tyrosine kinase activity of JAK2. IL-2 and IL-4 activate the tyrosine phosphorylation of JAK1 and of an additional 116-kDa putative JAK kinase family member.

Other investigators expressed the EPO-R in CTLL cells and obtained conflicting results. In one report (65), transfection of the EPO-R cDNA resulted in cell surface expression of EPO-R but no EPO-induced mitogenesis. Other groups have demonstrated that EPO activates JAK2 tyrosine phosphorylation in CTLL-EPO-R cells but does not activate mitogenic signaling (24). Our CTLL subclone may express an additional, accessory EPO-R subunit or functional downstream signaling molecule that is recruited by activated JAK2. Alternatively, the level of JAK2 expression in our CTLL subclone may account for these discrepant results. Similarly, the IL-4 phosphorylated substrate, 4PS, is expressed at different levels in FDC-P2 and 32D cells (59).

Our data provide evidence that the 122-kDa tyrosine-phosphorylated protein, activated by IL-2 or IL-4, is JAK1. This protein migrates with a slightly slower mobility than JAK2, consistent with published reports (55). The migration of JAK1 identified in R80 immune complexes is identical to authentic JAK1, as shown in experiments using the peptide-specific JAK1 antiserum. It is not immunoreactive with anti-JAK2 or anti-Tyk2.

We also tested another JAK1 antibody (UBI 06-254) and failed to detect JAK1 tyrosine phosphorylation (data not shown). This antibody is unable to detect tyrosine-phosphory-

lated JAK1 in immunoblotting experiments. Therefore, studies using this antibody (54, 64) require reinterpretation.

The lower p116 band that is tyrosine phosphorylated in response to IL-2 and IL-4 appears to be a distinct member of the JAK kinase family. p116 immunoprecipitated with antibody R80 but did not immunoprecipitate with the peptide-specific JAK1 antiserum. p116 is not a proteolytic fragment of JAK1, since it is not detected on Western blots (immunoblots) with an anti-JAK1 antiserum raised against an internal epitope (amino acids 785 to 804 of JAK1) (Fig. 4B, JAK1 immunoblot, lane 5). However, p116 does immunoprecipitate with JAK1 after IL-2 or IL-4 stimulation when cells are lysed with Brij 96; these complexes are disrupted in Triton X-100. Alternatively, p116 could represent an alternatively spliced JAK1. p116 is also not immunoprecipitated with anti-JAK2 or anti-Tyk2. Antiphosphotyrosine blots of whole cell lysates demonstrate that p116 is a prominent tyrosine-phosphorylated substrate after IL-2 (26) or IL-4 (25, 58) stimulation, consistent with earlier reports.

This is the first evidence that JAK tyrosine kinases are activated by IL-2 and IL-4. The IL-2-R β (4, 15, 36, 52), IL-2-R γ (2), and IL-4-R (25, 58) are all tyrosine phosphorylated upon activation, and several substrates are also tyrosine phosphorylated. Earlier experiments have provided evidence that Src family kinases are activated by IL-2 stimulation and associate with an acidic region of the IL-2-R β cytoplasmic tail (21, 27, 37). Functional redundancy was observed, as various Src kinases were activated in cell lines from different lineages (27, 46, 56). Activation of Src kinase activity may be specific to the IL-2 signaling pathway, as Src kinase association with the IL-4 receptor has not been demonstrated to date. On the other hand, activation of JAK tyrosine kinase activity may account for the tyrosine phosphorylation of common substrates by IL-2 and IL-4.

In this study, we have shown that IL-2 and EPO activate distinct JAK tyrosine kinases in the same cellular background. We would predict that receptor stimulation with other cytokines of the IL-2 family, namely, IL-7, IL-13 (35, 38), and IL-15 (17), would result in identification of a similar tyrosine-phosphorylated doublet after immunoprecipitation with R80. The use of the anti-JAK cross-reactive antibody (R80) described in this report may facilitate the rapid enumeration of JAK tyrosine kinases activated by specific cytokines.

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ADDENDUM IN PROOF

A cDNA for p116 has been isolated and is now called JAK3 [T. Takahashi and T. Shirasawa, *FEBS Lett.* **342**:124-128, 1994; M. Kawamura, D. W. McVicar, J. A. Johnston, T. B. Blake, Y.-Q. Chen, B. K. Lal, A. R. Lloyd, D. J. Kelvin, J. E. Staples, J. R. Ortaldo, and J. J. O'Shea, *Proc. Natl. Acad. Sci. USA* **91**:6374-6378, 1994; and B. A. Witthuhn, O. Silven-

nainen, O. Miura, K. S. Lai, C. Curik, E. T. Liu, and J. N. Ihle, *Nature (London)* **370**:153–157, 1994].

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