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Erythropoietin Induces Activation of Stat5 through Association with Specific Tyrosines on the Receptor That Are Not Required for a Mitogenic Response

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The cytoplasmic domain of the erythropoietin receptor (EpoR) contains a membrane-distal region that is dispensable for mitogenesis but is required for the recruitment and tyrosine phosphorylation of a variety of signaling proteins. The membrane-proximal region of 96 amino acids is necessary and sufficient for mitogenesis as well as Jak2 activation, induction of c-*fos*, c-*myc*, *cis*, the T-cell receptor γ locus (TCR-γ), and c-*pim-1*. The studies presented here demonstrate that this region is also necessary and sufficient for the activation of Stat5A and Stat5B. The membrane-proximal domain contains a single tyrosine, Y-343, which when mutated eliminates the ability of the receptor to couple Epo binding to the activation of Stat5. Furthermore, peptide competitions demonstrate that this site, when phosphorylated, can disrupt Stat5 DNA binding activity, consistent with a role of Y-343 as a site of recruitment to the receptor. Cells expressing the truncated, $Y^{343}F$ mutant (a mutant with a Y-to-F alteration at position 343) proliferate in response to Epo in a manner comparable to that of the controls. However, in these cells, Epo stimulation does not induce the appearance of transcripts for *cis*, TCR-γ, or c-*fos*, suggesting a role for Stat5 in their regulation.

Erythropoietin (Epo) regulates the proliferation and differentiation of cells of the erythroid lineage (25). Epo functions through its interaction with a single chain, Epo-specific receptor (EpoR), of the cytokine receptor superfamily (4, 9). Members of the cytokine receptor superfamily couple ligand binding to the induction of cellular protein tyrosine phosphorylation through their interaction with members of the Janus protein tyrosine kinase (Jak) family (23). EpoR specifically associates with Jak2 through the receptor cytoplasmic, membrane-proximal domain containing the conserved box 1 and box 2 motifs found in a number of the members of the cytokine receptor superfamily. Mutations or deletions in the membrane-proximal domain which affect Jak association affect all receptor functions that have been examined.

Epo, like a number of cytokines, activates a variety of signaling pathways. Previous studies have shown that the cytoplasmic membrane-distal region of the receptor is required for recruitment and tyrosine phosphorylation of SHC and the subsequent activation of the ras pathway (33). The membrane-distal region is also required for Epo-induced tyrosine phosphorylation of the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (8, 32) and phospholipase C-γ1 (40). The membrane-distal region is also required for the association of hematopoietic cell phosphatase (59), which negatively influences the receptor complex (44, 58, 59) and is hypothesized to be essential to terminate a response. Other potential signaling reactions detected in response to Epo include Syp (46), c-cbl (35), and Vav phosphorylation (31).

In addition to the above-described signaling pathways, a number of cytokines activate members of the family of signal transducers and activators of transcription (Stat). Although this transcription factor family was initially identified in interferon (IFN) responses (12), most cytokines that utilize receptors of the cytokine receptor superfamily have been found to activate one or more of the Stat family members. The family currently consists of Stat1 and Stat2, which were identified to act in IFN signaling; Stat3 (1, 64), which is involved in interleukin 6 (IL-6) signaling; Stat4 (56, 63), which has recently been shown to be activated in response to IL-12 (3, 24); Stat5, which was initially identified in prolactin responses (48) but is activated by several cytokines (2, 27); and Stat6 (21, 38), which is involved in IL-4 responses. Recent studies have indicated that among these, Stat5 is activated by Epo (16, 49). In this work, we evaluate the ability of Epo to activate Stat5A and/or Stat5B in various cell lines and explore the domains of the receptor that are required for activation. Importantly, we demonstrate that although receptor Y-343 is critical for activation of Stat5 in response to Epo, it is not required for a mitogenic response.

MATERIALS AND METHODS

Cell lines. All mammalian cell lines used were maintained in RPMI 1640 containing 10% fetal bovine serum and supplemented with appropriate growth factors. Murine DA-3 or 32Dcl3 cells expressing wild-type or mutant human EpoRs (28, 29) were maintained in media supplemented with murine IL-3 (25 U/ml). Murine CTLL cells were engineered to express EpoR by transfection with the human EpoR cDNA (10) in the expression vector pXM and were maintained in media supplemented with human IL-2 (25 ng/ml). Human TF-1 cells (American Type Culture Collection) were maintained in media supplemented with 10 ng of human IL-3 per ml. Sf9 cells were maintained in Grace's Insect Media supplemented with 10% fetal bovine serum.

Antisera and antibodies. Antisera specific for Stat3, Stat4, Stat6, Jak2, and Jak3 have been described previously (39, 53, 54). Antiserum recognizing both Stat5A and Stat5B was prepared by immunizing rabbits with a synthetic peptide corresponding to amino acids 14 to 25 of Stat5A (GDALRQMQVLYGQH). Antiserum specific for Stat5A was prepared by immunizing rabbits with a synthetic peptide corresponding to the unique carboxy-terminal domain of Stat5A (LDARLSPPAGLFTSARSSLS). Antiserum specific for Stat5B was obtained commercially (Santa Cruz Biotechnologies). Antibodies against Stat1 and Stat2

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were obtained from Transduction Laboratories. Antiphosphotyrosine antibodies (PY20) were obtained from ICN.

Mutagenesis and cDNA expression. EpoR-H/Y^{343}F was constructed by site-directed mutagenesis by using the PCR as previously described (19). Briefly, complementary oligonucleotides which incorporated a single adenine-to-thymidine mutation at nucleotide 1127 of the EpoR cDNA were synthesized. These oligonucleotides were used in combination with flanking primers for first-step PCR using the EpoR-H cDNA as a template. The products of first-step PCR were purified and mixed for use in a second-step PCR using only the flanking primers. The final product was then sequenced and cloned into the pXM vector. The EpoR-H/Y^{343}F cDNA in the pXM vector was coelectroporated into DA3 cells with the selectable marker pSV2neo. Transfected cells were selected in bulk in media containing murine IL-3 (25 U/ml) and G418 (1 mg/ml) for 14 days. Clonal populations of transfected cells were then obtained by limiting dilution in media containing IL-3 and G418. Individual clones were then assayed for their ability to proliferate in the presence of Epo or for the ability of Epo to stimulate cellular events associated with receptor activation.

A murine full-length Stat5A cDNA was isolated by screening a MEL cell library (Clontech) with a ^{32}P-labeled PCR fragment predicted by amino acid sequences obtained from purified murine Stat5 protein (2). A full-length murine Stat5B cDNA was kindly provided by Alice Mui (DNAX). For expression in COS-7 cells, the Stat5A cDNA in the expression vector pRK5 or Stat5B cDNA in the expression vector pME18S was transfected into COS-7 cells by using LipofectAmine (Life Technologies) as directed by the manufacturer. Cells were harvested for analysis 60 h following transfection.

Immunoprecipitation, SDS-PAGE, and Western blotting (immunoblotting). Prior to stimulation, cells were starved for 14 to 16 h in RPMI 1640 supplemented with 1% fetal bovine serum. Starved cells were treated with growth factor and were subsequently lysed in lysis buffer (1% Triton X-100, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, 5 mM EDTA, 0.1% bovine serum albumin [BSA], 0.05 mg of phenylmethylsulfonyl fluoride [PMSF] per ml, 10 mM Tris [pH 7.6]). Lysates were cleared of debris at 12,000 × *g* for 10 min, and the supernatants were incubated in the presence of the designated sera for 2 h. Immune complexes were precipitated with protein A-Sepharose (Sigma) and extensively washed in lysis buffer without BSA, and proteins were then eluted with sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Eluted proteins were separated on 7% polyacrylamide gels and transferred to nitrocellulose. Filters were probed with designated sera or antibodies and visualized with the ECL detection system (Amersham) as directed by the manufacturer.

Northern (RNA) blot analysis. Total cellular RNA was isolated from cells by acidic guanidinium thiocyanate-phenol-chloroform extraction (6). A 25-μg amount of total RNA was separated on 1.2% agarose–formaldehyde gels by electrophoresis and blotted onto nylon filters. The filters were hybridized with ^{32}P-labeled, randomly primed cDNA fragments, and detection was performed by autoradiography. The DNA probes used were a 200-bp PCR fragment from the 5′ end of the mouse cytokine-inducible SH2-containing protein (CIS), a 950-bp *Xba*I-*Sst*I mouse c-*myc* exon 2 fragment, a 1.6-kbp *Eco*RI fragment from rat c-*fos*, and a 1.3-kbp *Eco*RI fragment of the murine T-cell receptor γ (TCR-γ) locus.

Stat5 purification and in vitro phosphorylation. Purification of Stat5 from Sf9 cells was performed as previously described for Stat1 (39). Briefly, Sf9 cells infected with Stat5A virus were lysed by sonication in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6)–1 mM EDTA–5 μg of PMSF per ml–10% glycerol. Lysates were separated by sequential chromatography over a DEAE-Sepharose column (Pharmacia) and a Sephacryl S-300 column (Pharmacia). Immune complex Jak2 was prepared for infected Sf9 cells as described previously (39).

For in vitro kinase reactions, immune complex Jak2 was washed in kinase buffer (10 mM HEPES [pH 7.6], 50 mM NaCl, 100 μM Na_3VO_4, 5 mM $MnCl_2$, 5 mM $MgCl_2$) and mixed with purified Stat5 (1 μg of protein) plus 5 mM ATP or [γ-^{32}P]ATP. Following 30 min of incubation at 25°C, reactions were stopped by the addition of EDTA to a 15 mM final concentration and the soluble portion of the reaction mixture was isolated by centrifugation. The products of these reactions were analyzed either by separation on SDS-PAGE gels and autoradiography or, for DNA binding activity, by gel mobility shift analysis.

Gel mobility shift assays. Following the appropriate treatment, cells were lysed in 0.5% Nonidet P-40–50 mM Tris (pH 8.0)–0.1 mM EDTA–150 mM NaCl–100 μM Na_3VO_4–50 mM NaF, 1 mM dithiothreitol–0.4 mM PMSF–3 μg of aprotinin per ml–2 μg of pepstatin A per ml–1 μg of leupeptin per ml–10% glycerol. Cell extracts (10 μg of total protein) were incubated with 2 μg of poly(dI-dC) for 30 min and then with 1 ng of Klenow fragment-labeled probe DNA for another 30 min. The probe used (5′-CTAGCAGTGTTTCCCCGAAA CACGCTAG-3′) contains a core sequence corresponding to the GAS element found in the IRF-1 promoter. Samples were run on a 4.5% polyacrylamide gel in 200 mM Tris-borate–2.2 mM EDTA. Gels were dried and visualized by autoradiography. For peptide competition of GAS binding activity, cell extracts were incubated for 30 min at 25°C with 200 μM synthetic peptide prior to incubation with poly(dI-dC) and probe.

RESULTS

Epo specifically induces the activation of Stat5A and Stat5B. We initially examined the ability of Epo to induce the tyrosine phosphorylation of the cloned Stat family members. As illustrated in Fig. 1A, Stat1, Stat3, Stat4, Stat5, and Stat6 were all expressed in the DA-3 myeloid cell line examined, while only low levels of Stat2 were detectable by immunoprecipitation and Western blotting. Following stimulation of the cells with Epo for 20 min, there was a readily detectable induction of tyrosine phosphorylation of Stat5. In contrast, the other Stat proteins were not detectably phosphorylated. Similar results have been obtained with other myeloid cell lines transfected with the EpoR, including 32Dcl3 and BaF3 cells (data not shown). Stat activation by Epo was also examined by the induction of GAS binding activity. With an oligonucleotide containing the IRF-1 GAS sequence, Epo induces the appearance of a single binding complex in both DA-3 and 32Dcl3 cells expressing the EpoR (Fig. 1B). These complexes were completely supershifted with an antiserum against Stat5 but were not altered by antisera against Stat1, Stat3, Stat4, or Stat6 (data not shown).

In mice, Stat5 activity is encoded by two highly related, chromosomally linked genes (the Stat5A and Stat5B genes) (2, 7, 34), which are not distinguished by the antiserum used in the experiments shown in Fig. 1A. To determine whether activation of Stat5A and/or Stat5B was induced by Epo, Stat5A and Stat5B antisera specific to the unique C-terminal regions were utilized. The specificities of the antisera are shown in Fig. 2A. For the initial characterizations, cDNAs for Stat5A or Stat5B were expressed in COS cells and the Stat proteins were precipitated with the individual antisera and Western blotted with the antisera. As illustrated in Fig. 2A, Stat5A was specifically immunoprecipitated and Western blotted by the antiserum against Stat5A. Conversely, the Stat5A antiserum did not detectably immunoprecipitate Stat5B. Similarly, the Stat5B antiserum immunoprecipitated Stat5B and Western blotted Stat5B. Stat5A was not detected in immunoprecipitates with antiserum against Stat5B. Therefore, each of the Stat5 antisera is specific for its respective protein.

The above-described antisera were used to examine the Epo-induced tyrosine phosphorylation of Stat5A and Stat5B in DA-3 cells. As illustrated in Fig. 2B, Stat5A was inducibly tyrosine phosphorylated in response to Epo stimulation. Epo also induced tyrosine phosphorylation of proteins detected by the Stat5B antiserum at levels comparable to that seen with the antiserum against Stat5A, suggesting that Stat5B was also inducibly tyrosine phosphorylated. We reasoned that Stat5A and Stat5B would form heterodimers if both were inducibly tyrosine phosphorylated; therefore, to further explore the utilization of Stat5A and StatB, cells were induced with Epo, the complexes were immunoprecipitated with the Stat5B-specific antiserum, and the presence of Stat5A in the complexes was examined by Western blotting. As illustrated in Fig. 2C, there was no detectable Stat5A coimmunoprecipitated with Stat5B from unstimulated cells. However, following stimulation with Epo, Stat5A was readily detectable in immunoprecipitates obtained with the Stat5B antiserum, indicating the presence of heterodimers of Stat5A and Stat5B in stimulated cells.

Requirement for Jak2 activation for Stat5 activation. We have previously shown that Stat1 is a direct substrate for the Jaks in in vitro reactions with purified proteins (39). To determine whether Stat5 is also a substrate for Jak2, similar experiments were done. Stat5 was produced in insect Sf9 cells with a baculovirus expression construct, and the protein was purified as previously described for Stat1 (39). Immune complex-

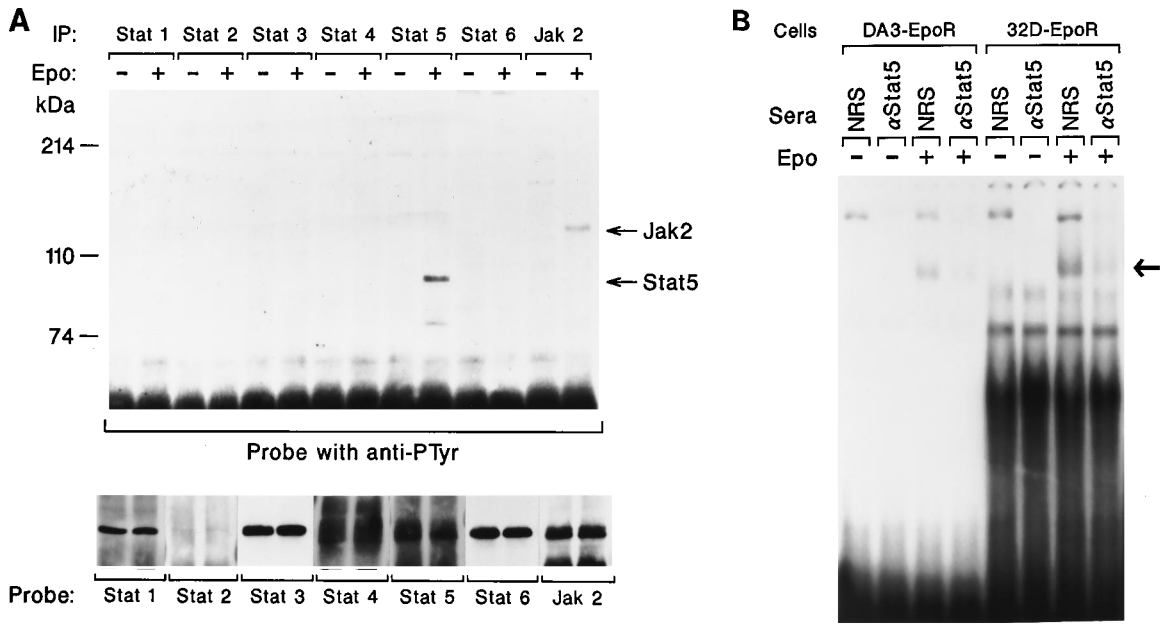

FIG. 1. Stat5 is phosphorylated and activated in response to Epo. (A) DA-3 cells transfected with EpoR were either not treated with factor (−) or treated for 20 min with 10 U of Epo per ml (+). Cell lysates then were immunoprecipitated (IP) with antiserum for Stat1, Stat2, Stat3, Stat4, Stat5, Stat6, or Jak2. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine (anti-P-Tyr). Subsequently, filters were stripped and reprobed with antiserum for each Stat protein and Jak2. (B) DA-3 cells expressing the EpoR (DA3-EpoR) or 32Dcl3 cells expressing the EpoR (32D-EpoR) were lysed after no stimulation (−) or following 20 min of treatment with 10 U of Epo per ml (+). The cell extracts were preincubated with either normal rabbit serum (NRS) or anti-Stat5 serum (1.5 μl) and were then subjected to a gel mobility shift assay as described in Materials and Methods. The Epo-induced DNA binding complex is indicated by the arrow.

purified Jak2 was obtained from Sf9 cells. As illustrated in Fig. 3A, purified Stat5 became highly phosphorylated when incubated in the presence of ATP and Jak2. No phosphorylation was seen in the absence of Jak2. In order to assess the effect of Jak2-mediated phosphorylation on DNA binding activity, the products of the in vitro reactions were subjected to gel mobility shift analysis with a consensus GAS oligonucleotide. As shown in Fig. 3B, GAS binding activity was readily detectable in reaction mixtures containing Stat5 and Jak2 but not in reaction mixtures containing only Stat5.

To address the question of whether Stat5 is a direct substrate of Jaks in vivo, we examined the correlation of Stat5 activation with Jak2 activation among a series of point mutants altered within the membrane-proximal domain of the cytoplasmic domain of the EpoR. The mutants examined, along with their ability to activate Jak2, are indicated in Fig. 4. Of particular importance, two point mutations, $Q^{280}L$ (a Q-to-L alteration at position 280) and $W^{282}R$, which dramatically reduced the ability to activate Jak2, also dramatically reduced the induction of Stat5 GAS binding activity. Conversely, mutations that did not affect Jak2 activation did not affect the induction of GAS binding activity. Lastly, a mutation in the extracellular domain of the EpoR (51) has been previously shown to cause the constitutive, ligand-independent activation of the receptor and Jak2 (31). This mutation conferred on the cells a constitutive, ligand-independent activation of Stat5 GAS binding activity. Together, the results are consistent with the hypothesis that Jak2 is activated and phosphorylates Stat5A and Stat5B following Epo stimulation.

Role of receptor tyrosines in Stat5 activation. The recruitment of Stat proteins to various receptor complexes has been shown to be dependent upon the interaction of the SH2 domains of Stat proteins with specific tyrosine residues within the cytoplasmic domains of the receptors (18, 45). To determine the domains of the EpoR that are required for activation of Stat5, we utilized receptors with carboxyl-terminal truncations and an internal deletion. As shown in Fig. 5, no Stat5 tyrosine phosphorylation was detected with the internal deletion mutant (PB) or with the S carboxyl-terminal truncation mutant. Both of these mutations have previously been shown to eliminate Jak2 activation and a mitogenic response (53). In contrast, the mitogenically active H carboxyl-terminal truncation mutant retained the ability to induce Stat5 tyrosine phosphorylation.

The H carboxyl-terminal truncation mutant retains a single Y residue that is not present in the S carboxyl-terminal truncation mutant which could be involved in the recruitment of Stat5 to the receptor complex. However, it should be noted that in previous studies we have not detected tyrosine phosphorylation of this mutant receptor (30). To explore the possible involvement of Y-343 in Stat5 activation, a mutant receptor that contained the H carboxyl-terminal truncation and a $Y^{343}F$ point mutation was made. This mutant was transfected into DA-3 cells, and individual clones were selected in IL-3. The clones were then examined for Epo induction of the tyrosine phosphorylation of Jak2 and Stat5. As shown in Fig. 6, no phosphorylated Stat5 was detected following Epo stimulation of cell lines expressing EpoR-H/$Y^{343}F$. Although the degree of Epo-induced Jak2 phosphorylation varied among clones, cell lines in which Epo and IL-3 induced comparable Jak2 activation failed to show detectable levels of Stat5 phosphorylation following Epo stimulation. Therefore, Y-343 is critical for Stat5 activation, although whether phosphorylation of Y-343 is required could not be established.

The activation of the DNA binding activity of Stat proteins is dependent upon dimerization through interaction of the SH2 domain with carboxyl sites of phosphorylation (43). Activated dimers can be disrupted by phosphopeptides that are

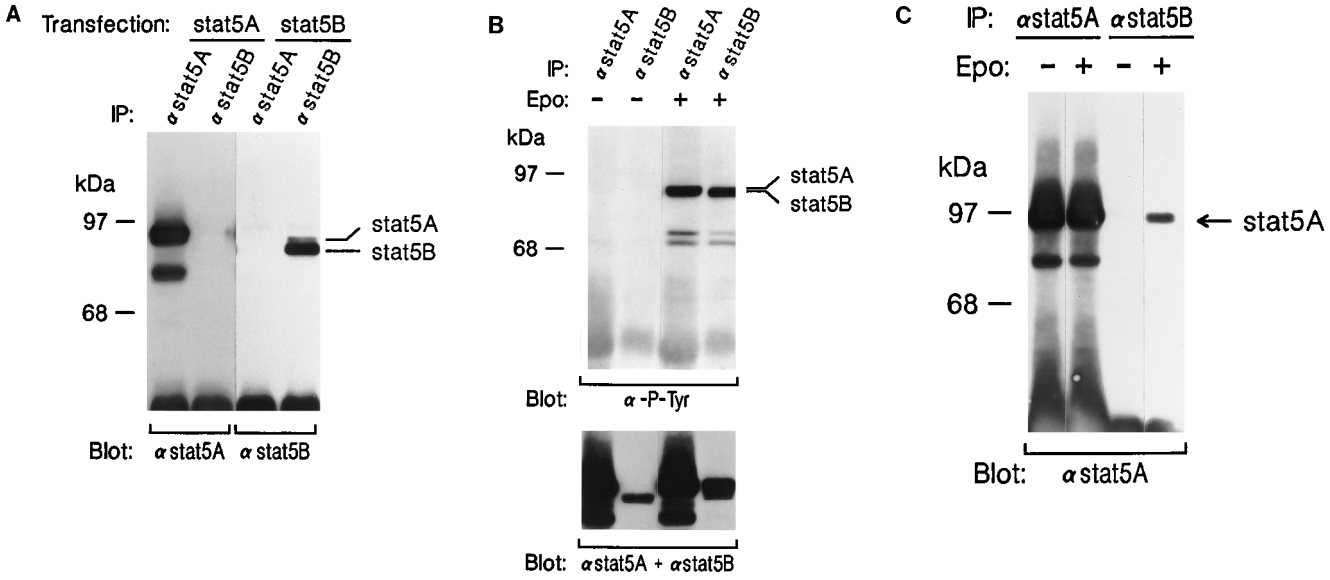

FIG. 2. Epo-induced phosphorylation of both Stat5A and Stat5B. (A) The specificities of antisera generated against peptides derived from the unique C-terminal ends of Stat5A and Stat5B were assessed by using COS cells transfected with expression constructs for either Stat5A or Stat5B. Lysates from each transfection were immunoprecipitated (IP) with Stat5A- or Stat5B-specific sera, and precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. Immunoprecipitated proteins from Stat5A-transfected cells were probed with the Stat5A-specific sera. Immunoprecipitated proteins from the Stat5B-transfected cells were probed with the Stat5B-specific sera. (B) Epo-induced phosphorylation of Stat5A and Stat5B was also assessed. 32Dcl3 cells expressing EpoR were stimulated with 10 U of Epo per ml (+) or left untreated (−). Cell lysates were prepared and immunoprecipitated (IP) with Stat5A- or Stat5B-specific sera. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine antibodies (α-P-Tyr). Subsequently, the blot was stripped and reprobed with both Stat5A and Stat5B sera. (C) In order to assess complex formation between Stat5A and Stat5B, cells were treated as described for panel B. Proteins immunoprecipitated (IP) with either Stat5A- or Stat5B-specific sera were probed with Stat5A-specific sera.

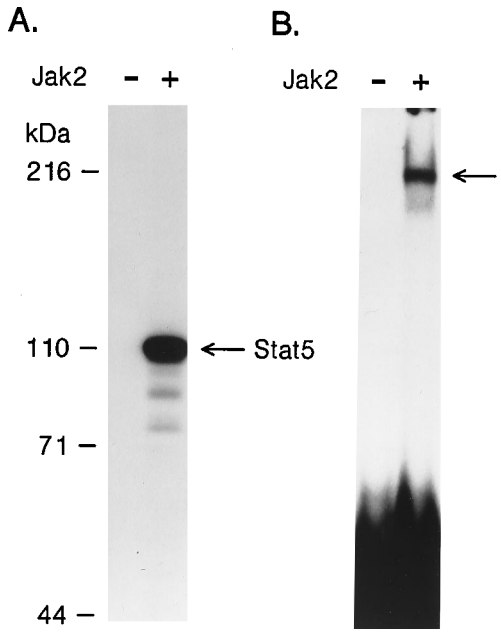

FIG. 3. Jak2-dependent in vitro phosphorylation and activation of Stat5. Stat5 expressed in Sf9 cells was purified as described in Materials and Methods. Purified Stat5 (1 μg) plus [γ-^{32}P]ATP was incubated with (+) or without (−) immune complex Jak2. (A) The soluble fraction of each reaction mixture was separated by SDS-PAGE and visualized by autoradiography. (B) Alternatively, products from parallel reactions performed with ATP were subjected to gel mobility shift analyses as described in Materials and Methods.

capable of binding to the SH2 domain, as assessed by their ability to eliminate DNA binding activity. We therefore used this approach to define the potential binding sites of Stat5 on the EpoR. Lysates from Epo-stimulated DA-3 cells were either incubated with synthetic phosphopeptides corresponding to the potential phosphorylation sites of the EpoR (Table 1) or left untreated as a control (Fig. 7A). Alternatively, lysates from insect cells coexpressing Stat5 and Jak2 were used (Fig. 7B). The Stat5 binding activity was then assessed by gel shift mobility assays. Only the phosphopeptides corresponding to Y-343, Y-431, and Y-479 disrupted Stat5 binding activity from DA-3 and insect cells. When these peptides were dephosphorylated (data not shown), the ability to disrupt Stat5 binding was significantly reduced, indicating that disruption of Stat5 binding activity requires the phosphotyrosine for high-affinity binding. To specifically assess the role of tyrosine phosphorylation of Y-343, a nonphosphorylated peptide was synthesized. As illustrated in Fig. 7A and B, this peptide did not disrupt Stat5 DNA binding activity.

As a control for specificity and correlation with cellular responses, we also examined the ability of the phosphopeptides derived from the EpoR to disrupt the DNA binding activity of Stat1. As shown (Fig. 7C), only the peptide containing Y-431 disrupted Stat1 DNA binding activity. Binding also required phosphorylation of the tyrosine (data not shown), indicating that this is a potential binding site for Stat1 on the EpoR. However, as shown in Fig. 1, we have not detected Epo-induced tyrosine phosphorylation of Stat1. Whether this reflects the lack of phosphorylation of this site in cells or a relatively lower, and not physiologically relevant, affinity of Stat1 for this site is not known.

Lack of a requirement for Stat5 in Epo-induced proliferation. The potential role of Stat5 in the response of cells to Epo was assessed by examining cell lines containing the various

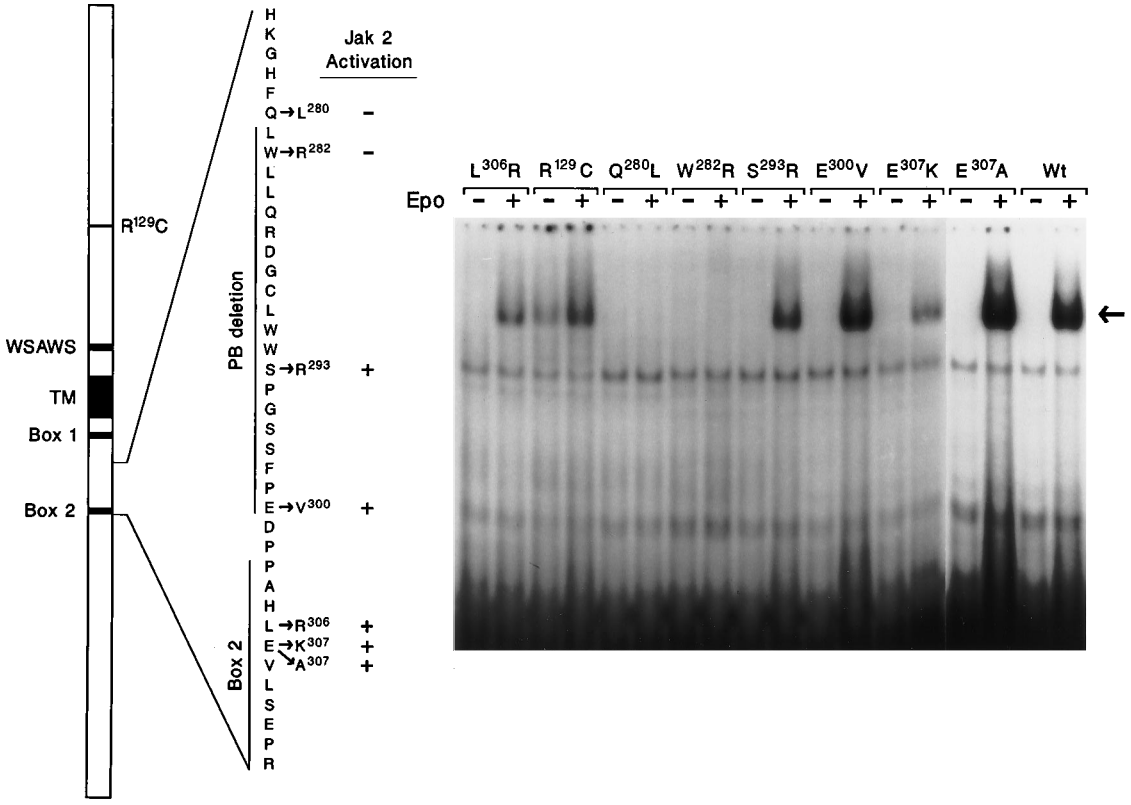

FIG. 4. Stat5 activation correlates with Jak2 activation in EpoR point mutants. Point mutations made within the extracellular and cytoplasmic, membrane-proximal domains of EpoR are diagrammed on the left, and their abilities to activate Jak2 are indicated. The transmembrane domain (TM) and conserved WSAWS, box 1, and box 2 domains are also indicated. DA-3 cells expressing these mutant receptors were either not treated with factor (−) or treated with 20 U of Epo per ml (+) for 20 min. Lysates were then prepared and subjected to gel mobility shift analysis. The Epo-induced DNA binding complex is indicated by the arrow.

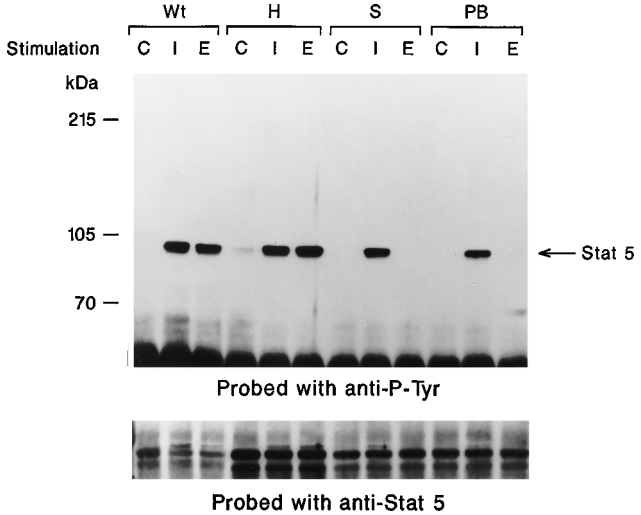

FIG. 5. The membrane-proximal cytoplasmic domain of EpoR is required for Stat5 phosphorylation. DA-3 cells expressing wild-type EpoR (Wt), EpoR truncated at amino acid 377 (H), EpoR truncated at amino acid 338 (S), or EpoR containing a deletion between amino acids 281 and 301 (PB) were either not treated with factor (C) or treated with IL-3 (I) or Epo (E). Cell lysates were then immunoprecipitated with antiserum for Stat5. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine (anti-P-Tyr). Subsequently, filters were stripped and reprobed with anti-Stat5 serum.

receptors. In DA-3 cells, the S carboxyl-terminal truncation mutant is not capable of a mitogenic response while the H carboxyl-terminal truncation mutant is mitogenically active, indicating that the sequence between these sites of truncation is important for proliferation (30). The inability of the S mutant to activate Jak2 further suggests that this region may be critical for appropriate association of the receptor with Jak2. To determine whether Y-343 was specifically involved in mitogenesis, the H carboxyl-terminal truncation mutant, containing the $Y^{343}F$ mutation, was transfected into DA-3 cells, individual clones were isolated in IL-3 and G418, and the clones were assessed for their ability to grow in Epo. As shown in Fig. 8, all the cell clones expressing the EpoR-H/$Y^{343}F$ mutant grew at comparable rates in the presence of Epo and IL-3 and the clones could be maintained long-term in Epo alone (data not shown).

Unlike the situation with myeloid lineage cells, expression of the EpoR in T-lineage cells does not allow Epo-induced proliferation (57). It was therefore of interest to determine whether the receptor retains the ability to couple ligand binding to activation of Stat5. For these experiments, the EpoR was electroporated into CTLL cells and individual clones were obtained by selection in the presence of IL-2 and G418. Consistent with previous results, none of the selected clones had the ability to proliferate in Epo (data not shown). The clones were next examined for the ability of Epo to induce the tyrosine phosphorylation of Jak2 as well as to induce Stat5 tyrosine phosphorylation. As illustrated in Fig. 9, Epo was capable of inducing the phosphorylation of both Jak2 and Stat5.

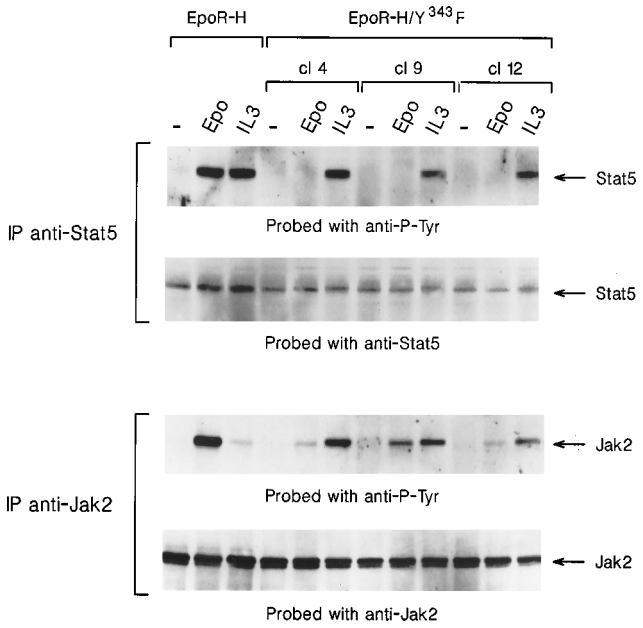

FIG. 6. Epo does not activate Stat5 in DA-3 cell lines expressing EpoR-H/$Y^{343}F$ receptors. DA-3 cells expressing EpoR-H or three subclones of DA-3 cells expressing EpoR-H/$Y^{343}F$ were either not treated with factor (−) or treated with Epo or IL-3. Cell lysates were then immunoprecipitated (IP) with antiserum for Stat5 or Jak2. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine (anti-P-Tyr). Subsequently, filters were stripped and reprobed with anti-Stat5 or anti-Jak2 serum.

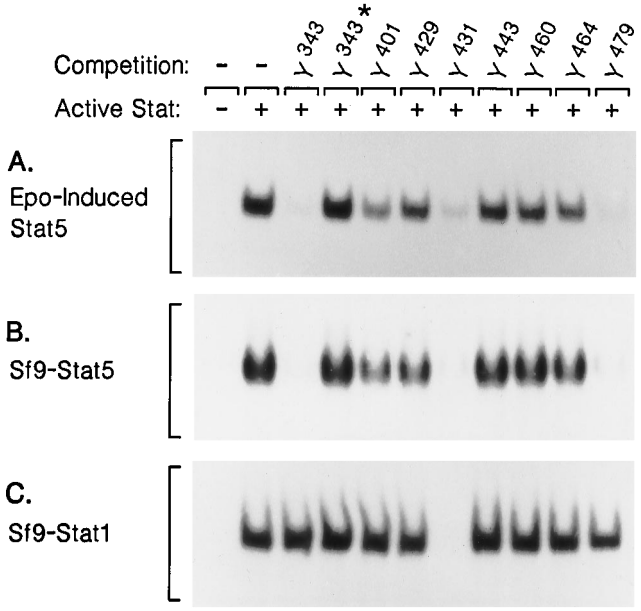

FIG. 7. EpoR-derived phosphopeptide competition of Stat5 DNA binding activity. (A) Lysates from DA-3 cells expressing EpoR either not treated with factor (−) or treated with Epo (+) were preincubated with or without synthetic phosphopeptides (200 μM) corresponding to potential tyrosine phosphorylation sites of EpoR (Table 1). The nonphosphorylated equivalent of the Y-343 peptide (Y^{343*}) was also used in competition experiments. Lysates were then assayed for DNA binding activity by a gel mobility shift assay. (B and C) Alternatively, Sf9 cells were infected with baculovirus expression vectors for Stat5 or Stat1 (−) or were coinfected with Stat5 and Jak2 or Stat1 and Jak2 viruses (+). 72 h after infection, cell lysates were prepared and assayed as described for panel A.

Thus, within the context of the CTLL cells studied the activation of Stat5 is not sufficient for a mitogenic response.

A variety of cell lines that can proliferate in response to Epo have been established. We therefore examined a number of these cell lines to determine whether Epo consistently induced the tyrosine phosphorylation of Stat5. Although Stat5 induction was observed to occur in almost all cell lines examined, no induction of Stat5 phosphorylation was seen in the TF-1 cell line (Fig. 10). However, the cells contain Stat5, and its phosphorylation could be induced by IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF). Importantly, TF-1 cells proliferate comparably in response to both Epo and IL-3 (data not shown).

Dependence of *cis*, TCR-γ, and c-*fos* induction but not c-*myc* expression on Y-343. Cytokines, including Epo, induce the transcription of several immediate-early genes (28), including a newly identified gene termed the cytokine-inducible SH2-containing-gene (*cis*) (60). We therefore examined the ability of Epo to induce gene expression in cells containing the EpoR-H or the EpoR-H/$Y^{343}F$ receptors to identify those genes that may be under the specific regulation of Stat5. As illustrated in Fig. 11, the $Y^{343}F$ mutation did not detectably affect the ability of the receptor to couple ligand binding to the induction of appearance of transcripts for c-*myc*, although with the background level of expression of c-*myc* seen in this clone, the fold induction was only two- to threefold. However, the H/$Y^{343}F$ mutation eliminated Epo-induced appearance of transcripts for *cis*. We have previously shown that the H mutant retains the ability to couple Epo binding to induction of c-*fos* and TCR-γ transcripts (28). As illustrated in Fig. 11, the H/$Y^{343}F$ mutant was also unable to couple Epo binding to the appearance of c-*fos* or TCR-γ transcripts, consistent with a potential role of Stat5.

TABLE 1. Sequences of phosphotyrosine peptides from EpoR

Amino acid sequence	Competition for[a]:	
	Stat5	Stat1
QDT Y-343 LVLDKWL	+	−
SFE Y-401 TILDPSS	−	−
HLK Y-429 LYLVVSD	−	−
KYL Y-431 LVVSDSG	+	+
STD Y-443 SSGGSQG	−	−
DGP Y-460 SHPYENS	−	−
SHP Y-464 ENSLVPD	−	−
HPG Y-479 VACS	+	−

[a] +, peptide able to disrupt binding; −, peptide unable to disrupt binding.

DISCUSSION

The activation of one or more members of the Stat family of transcription factors has been shown for a variety of cytokines that utilize receptors of the cytokine receptor superfamily. Consistent with previous results (16, 49), we find that Epo specifically induces the activation of Stat5, a Stat family member that was initially identified as a prolactin-inducible Stat involved in the regulation of the genes for milk proteins (62). Quite strikingly, Stat5 is inducibly tyrosine phosphorylated in responses to a variety of cytokines, including IL-3 (2, 27), GM-CSF (16, 27), IL-5 (27), IL-2 (14, 15, 22, 49), growth hormone (16, 55), and thrombopoietin (36), as well as to epidermal growth factor (41). Although Stat5 is clearly involved in prolactin-regulated expression of milk proteins in breast tis-

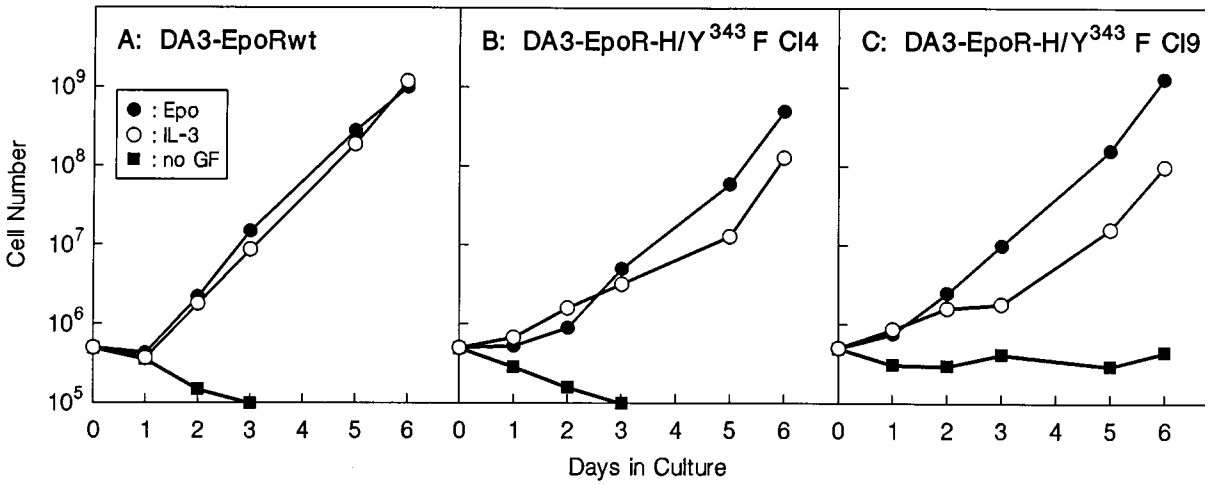

FIG. 8. DA-3 cell lines expressing EpoR-H/Y^{343}F receptors are factor dependent and proliferate in response to Epo. DA-3 cells expressing wild-type EpoR (DA3-EpoRwt) (A) or two subclones of DA-3 cells expressing EpoR-H/Y^{343}F (B and C) were washed free of growth factor and suspended in medium containing 5 U of Epo per ml (filled circles), 25 U of IL-3 per ml (open circles), or no factor (filled squares). Cell counts were performed for each culture over a period of 6 days, and the total number of cells present in the culture at each time point was calculated.

sues (26, 48), the significance of the activation of Stat5 in the other responses is unknown.

The specificity for the activation of Stat proteins in many receptor systems has been shown to reside in the receptor through the interaction of the Stat SH2 domain with sites of receptor tyrosine phosphorylation (18, 45). However, it should be noted that in the case of the growth hormone receptor, cytoplasmic tyrosine residues are not required for Stat activation (50). Our results indicate that multiple potential phosphotyrosine binding sites in the EpoR can be identified by their ability to disrupt Stat5 homodimers and eliminate DNA binding activity. Among these tyrosine residues, Y-343 is clearly important for the inducible tyrosine phosphorylation of Stat5 within the context of the truncated H receptor. Interestingly,

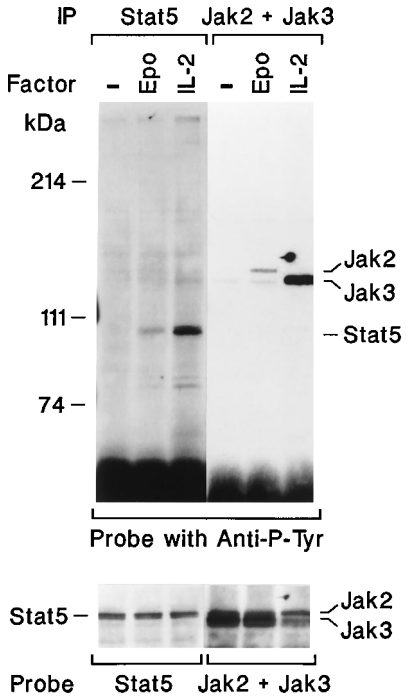

FIG. 9. Epo-induced phosphorylation of Stat5 in CTLL cells expressing EpoR. CTLL cells expressing EpoR were either not treated with factor (−) or treated for 20 min with Epo or IL-2. Cell lysates were then immunoprecipitated (IP) with antiserum for Stat5 or Jak2 and Jak3. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine (Anti-P-Tyr). Subsequently, filters were stripped and reprobed with anti-Stat5 or anti-Jak2-plus-anti-Jak3 serum.

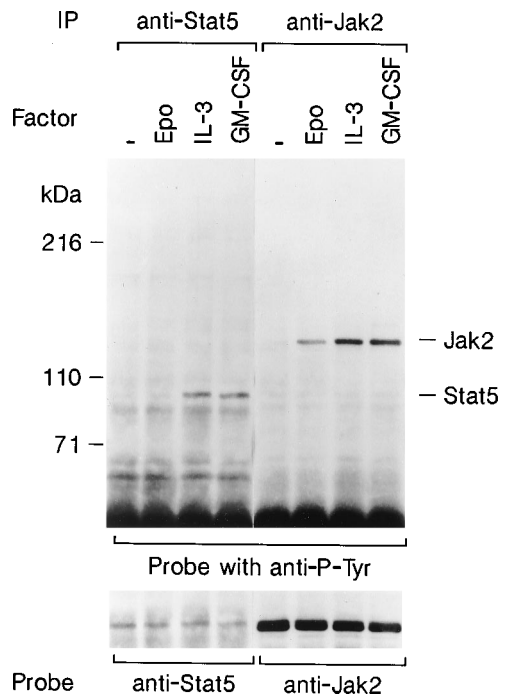

FIG. 10. Epo does not activate Stat5 in TF-1 cells. TF-1 cells were either not treated with factor (−) or treated for 20 min with Epo (10 U/ml), IL-3 (10 ng/ml), or GM-CSF (10 ng/ml). Cell lysates were then immunoprecipitated (IP) with antiserum for Stat5 or Jak2. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine (anti-P-Tyr). Subsequently, filters were stripped and reprobed with anti-Stat5 or anti-Jak2 serum.

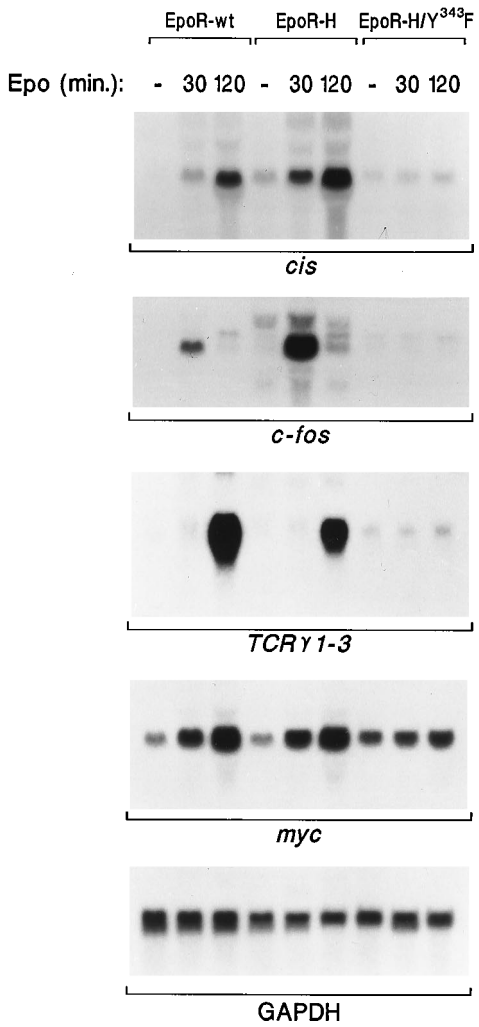

FIG. 11. Requirement for Stat5 for Epo-stimulated induction of immediate-early genes in DA-3 cells expressing EpoR. DA-3 cells expressing wild-type EpoR (EpoR-wt), EpoR-H, or EpoR-H/$Y^{343}F$ receptors were incubated in the absence of growth factor for 24 h and were then left untreated (−) or stimulated with 5 U of Epo per ml for 30 min or 2 h. Total RNA was extracted from cells and subjected to Northern analysis with the indicated probes as described in Materials and Methods. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

previous studies failed to identify tyrosine phosphorylation of the truncated H receptor, suggesting that Y-343 was not phosphorylated in cells. It is possible that this site, when phosphorylated, is not detected by the antiphosphotyrosine reagents that have been used. Alternatively it was possible that Stat5 recognizes a motif that includes Y-343 but does not require phosphorylation. We consider this unlikely because peptides containing Y-343 that is not phosphorylated do not effectively compete for Stat5 dimers.

Peptide competition experiments also identified Y-431 and Y-479 as potential binding sites for Stat5. Whether these sites are phosphorylated and whether they contribute to Stat5 activation in response to Epo are not known. Specifically, it will be necessary to make combinations of mutations of the three potential sites to assess their individual roles within the context of the full-length receptor. Interestingly, the peptide containing Y-431 was also capable of disrupting the DNA binding activity of Stat1, and this required its phosphorylation. However, Epo does not induce the tyrosine phosphorylation of Stat1. It is possible that Y-431 is not phosphorylated in cells. Alternatively, it is possible that the ability to disrupt DNA binding, at the concentration of peptide used, does not reflect affinities that are required for activation in cells.

The results demonstrate that in the H carboxyl-terminally truncated receptor, Y-343 is critical for Epo-induced activation of transcription of the *cis* gene. Previous studies (60) noted the existence of Stat5 binding sites in the promoter of the *cis* gene. Our results are consistent with the hypothesis that Stat5 regulates *cis* expression. However, it is quite possible that multiple signal-transducing proteins utilize Y-343, and their activity would also be compromised in cells expressing the mutant. The significance of Epo-induced *cis* expression is not known. The gene encodes a 37-kDa protein containing an SH2 domain which is capable of binding to phosphorylated cytokine receptors. Moreover, forced expression of *cis* was found to suppress cell growth (60). Thus, it was hypothesized that the CIS protein may control growth through sequestration of tyrosine-phosphorylated proteins. In this context, it should be reiterated that there was no detectable difference in the growth responses of cells expressing the H truncation or the H/$Y^{343}F$ truncation mutant that would be consistent with the possibility that induction of *cis* would suppress the growth response.

The ability of Epo to induce the transient appearance of transcripts for c-*fos* was retained by the H mutant, as previously reported (28), but was lost in the H/$Y^{343}F$ mutant, suggesting that c-*fos* induction may be under the control of Stat5. Previous studies identified a serum-inducible element (SIE) in the c-*fos* promoter (47) which binds cytokine- and growth factor-induced transcription factors of the Stat family (13, 42). However, recent studies have found that a simple correlation between a requirement for the SIE and induction of the activation of Stat proteins is not generally observed (20). It is possible that in hematopoietic cells, responding to certain cytokines, the Stat pathway is primarily involved in the regulation of c-*fos* expression.

As previously described, the H mutant also retained the ability to mediate Epo-induced expression of the TCR-γ locus transcripts (28). It should be noted, however, that the expression of TCR-γ locus transcripts is dependent upon synthesis of new protein (52) and therefore presumably requires the expression of another gene whose regulation may be directly under the control of signals from the EpoR. The lack of induction of the expression of TCR-γ transcripts by the H/$Y^{343}F$ mutant would suggest that Stat5 is involved in the expression of such a gene. The significance of Epo regulation of TCR-γ transcripts is not known; however, a number of cytokines induce expression and share in common the ability to activate Stat5. Among these, IL-7 is critical for the early lymphoid lineage and activates Stat5. Since transcription of unrearranged alleles is hypothesized to be critical for subsequent rearrangement, it could be hypothesized that transcription of the TCR-γ locus through Stat5 activation by IL-7 may contribute to the T-cell repertoire.

The mutation of Y-343 within the context of the H truncation also did not reduce the Epo-induced growth rate of the cells, suggesting that other Stat5-induced functions do not positively contribute to mitogenesis. It should be noted, however, that truncations within this region of the receptor have given variable results. Mutants with truncations at P-328 have been found to eliminate mitogenesis in all cases examined (5, 17). Mutants with truncations at H-337 have been reported to be mitogenically active (5). In our experience, this truncation is inactive in DA-3 cells (53) and is able to sustain proliferation in 32Dcl3 cells. In DA-3 cells, this mutant affects the ability of the receptor to couple ligand binding to activation of Jak2 (53).

Importantly, this mutant lacks Y-343 and would not be predicted to activate Stat5. In all studies examining the A-374 truncation, this mutant is found to be capable of a mitogenic response. Irrespective, because of the ambiguities of the consequences of truncations near Y-343, it is difficult to assess the role of Stat5 in the mitogenic response by using truncations.

Unexpectedly, we found that Epo did not induce Stat5 in TF-1 cells. It should be noted, however, that others have detected Stat5 activation in TF-1 cells (49). These differences may reflect a clonal variation that often characterizes hematopoietic cell lines. The basis for a cell type specificity for recruitment of Stat5 to the receptor complex is not known. One possibility is the cell stage-specific expression of SH2-containing proteins that can compete for the recruitment of Stat5 to the receptor complex. In this regard, it is interesting that the *cis* gene encodes a small SH2 domain-containing protein that binds to phosphorylated cytokine receptor complexes. The consequences of overexpression of *cis* on the ability of various receptors to recruit Stat proteins have not been examined.

The major conclusion from our studies is that mutations that uncouple a mitogenic response from activation of Stat5 can be identified, and thus our results suggest that other, presumably Jak2-dependent, pathways exist which are critical for a mitogenic response as well as for activations of critical genes such as c-*myc*. This is perhaps not unexpected, since mutations in a variety of receptors that eliminate Stat activation do not affect a mitogenic response, including IL-2-induced Stat5 activation (15), IL-4-induced Stat6 activation (38), and IL-6-induced Stat3 activation (45), and IFNs, which activate Stat1 and Stat2, are not mitogenic (12, 37). These conclusions are somewhat in conflict with recent reports which suggested that Stat proteins may play a role in cellular transformation (11, 61). However, both v-*abl* and v-*src* phosphorylate a variety of cellular substrates, and it is unknown which of these mediates the mitogenic response. Also, the experiments to date do not prove that the activations of Jaks or Stat proteins have any relevance to transformation.

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