MOLECULAR AND CELLULAR BIOLOGY, Oct. 1991, p. 4895–4902
0270-7306/91/104895-08$02.00/0

Vol. 11, No. 10

Induction of Tyrosine Phosphorylation by the Erythropoietin Receptor Correlates with Mitogenesis

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Received 17 April 1991/Accepted 8 July 1991

A role for tyrosine phosphorylation in the signal-transducing mechanisms of several hematopoietic growth factors has been hypothesized. To extend these observations, we have examined the effects of erythropoietin (Epo) on tyrosine phosphorylation in an Epo-responsive cell that was obtained by transfecting the murine erythropoietin receptor (EpoR) into an interleukin-3 (IL-3)-dependent cell line. By two-dimensional analysis of phosphotyrosine-containing proteins isolated with a monoclonal antibody (1G2) against phosphotyrosine, Epo and IL-3 were found to rapidly induce tyrosine phosphorylation of comparable substrates of 92, 70, and 56 kDa. In addition, Epo uniquely induced phosphorylation of a 72-kDa substrate while IL-3 uniquely induced phosphorylation of a 140-kDa substrate. Immunoprecipitation and mixing experiments indicated that the 72-kDa substrate may represent a small fraction of the EpoR. To explore the significance of tyrosine phosphorylation, we generated two mutants of the EpoR that lacked 108 or 146 amino acids at their carboxyl termini. In addition we constructed an internally deleted mutant that lacked 20 amino acids in a region of sequence homology with the IL-2 receptor β chain. Although all mutants were expressed at comparable levels and had comparable binding affinities for Epo, only the mutant lacking 108 amino acids at the carboxyl terminus retained significant mitogenic activity or the ability to induce tyrosine phosphorylation.

Erythropoietin (Epo) is a glycoprotein hormone that is the primary regulator of erythropoiesis (8, 11, 24). Following binding to specific cell surface receptors, Epo acts on committed erythroid progenitor cells by stimulating their proliferation and supporting their differentiation. The signal-transducing mechanisms in these responses are largely unknown, in part because of the difficulty of obtaining pure populations of Epo-dependent cells. Results of several studies using heterogenous populations of hematopoietic cells have suggested a role for cyclic AMP or changes in calcium levels (1, 27, 28, 36). However more recent studies, using highly enriched or pure populations of Epo-responsive cells, have failed to support a role for these mediators in proliferation (24). More recently, studies have demonstrated that Epo stimulation induces tyrosine phosphorylation. However, whether it is required for mitogenesis was not addressed (32).

In recent studies, cDNAs for the Epo receptor (EpoR) were isolated from an expression library from a mouse erythroleukemia cell line (6). When this receptor was expressed in an interleukin-3 (IL-3)-dependent pro-B-cell line (BA/F3), the cells proliferated in the presence of Epo, demonstrating the functionality of the cloned receptor. The nucleotide sequence of the receptor cDNA predicts a protein of 507 amino acids with a single membrane-spanning domain and motifs associated with the cytokine receptor superfamily. The cytoplasmic domain is devoid of recognizable motifs associated with kinases or other signal-transducing proteins. However, recent studies have identified two unique domains in the cytoplasmic tail (7). A membrane-proximal domain of ≤90 amino acids was required for signal transduction, while a carboxyl-terminal region of approximately 40 amino acids was a negative regulatory domain. Interestingly, the cytoplasmic domain of the EpoR has sequence similarity to the cytoplasmic region of the IL-2 receptor β chain (6). Furthermore deletional mutagenesis has shown that this region in the IL-2 receptor β chain is critical for mitogenesis (12).

In this study, we have examined the effect of Epo on tyrosine phosphorylation in an IL-3-dependent cell line transfected with an EpoR cDNA. In these cells, Epo induces the rapid appearance of tyrosine phosphoproteins that are similar to those seen following stimulation of the cells with IL-3 and a unique phosphoprotein of 72 kDa. Using a series of mutants, we have demonstrated a correlation between the ability to stimulate proliferation and to induce tyrosine phosphorylation. Deletion of 20 amino acids from the region of sequence similarity between the Epo and IL-2 β receptors completely eliminated both mitogenesis and tyrosine phosphorylation, further confirming the functional relatedness of these cytoplasmic regions.

MATERIALS AND METHODS

Cells and reagents. DA-3 cells, an IL-3-dependent-cell line derived from a primary Moloney murine leukemia virus-induced leukemia (15), were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 25 U recombinant murine IL-3 per ml. The DA-3 cell line was used because the cells do not express the EpoR and show no proliferation in response to Epo, and Epo-responsive variants have not been isolated. An expression plasmid for the murine EpoR, pXM-EpoR (6), and a rabbit polyclonal anti-peptide antiserum directed against the amino terminus of the murine EpoR (25) have been previously described. Recombinant human Epo was kindly provided by Amgen Biological (Thousand Oaks, Calif.). All other reagents used were purchased from commercial sources.

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Construction of expression plasmids for EpoR mutants. Plasmid pXM-ER-H was constructed by using the polymerase chain reaction (PCR) method (34). A portion of pXM-EpoR was amplified by the PCR method, using a 5′ primer corresponding to a sequence immediately upstream of the insertion site of the EpoR cDNA and a 3′ primer corresponding to a sequence at the *Hin*dIII site of the cDNA, with added sequences of a stop codon and an *Eco*RI recognition sequence. After digestion with both *Kpn*I and *Eco*RI, the PCR product was subcloned into the pXM vector digested with the same enzymes.

For the construction of pXM-ER-S, the full-length EpoR cDNA was subcloned into the *Kpn*I site of pUC18, and the 3′ region from the *Sph*I site was deleted by *Sph*I digestion. After blunt ending, an *Xba*I linker (Pharmacia) was used to introduce stop codons in all three reading frames. Because of a single-base deletion in the introduced linker, six amino acids were added. From the resulting plasmid, the truncated EpoR cDNA was cut out as a fragment having a *Kpn*I site and a blunt-ended *Hin*dIII site and inserted between the *Kpn*I site and the blunt-ended *Eco*RI site of the pXM vector.

For the construction of plasmid pXM-ER-PB, the *Kpn*I-*Pvu*II and *Bam*HI-*Kpn*I fragments of the EpoR cDNA in pXM-EpoR were fused and subcloned into pUC18 after the *Bam*HI site was blunt ended. The fused EpoR cDNA fragment, having the internal deletion of the *Pvu*II-*Bam*HI region, was then put back into the pXM vector as described above, except that the *Cla*I site in the 3′ noncoding region of the cDNA was used instead of the *Hin*dIII site in the multiple cloning region of pUC18. The structures of the expression plasmids were confirmed by digestion with multiple restriction enzymes and by DNA sequence analysis of appropriate regions.

Transfectants. Plasmid DNAs were transfected into DA-3 cells by electroporation. Samples (10 μg) of EpoR expression plasmids, linearized by *Pvu*I digestion, were cotransfected with 1 μg of *Eco*RI-linearized pSV2*neo*. Transfected cells were selected in medium containing G418 or in medium containing Epo instead of IL-3, and several subclones for each construct were isolated by limiting dilution or by colony formation in soft agar. Among the individual clones selected in either Epo or G418, all that were examined responded comparably to Epo. Selected subclones were then analyzed for the expression of the EpoR by ^{125}I-Epo binding assays, and the clone that bound the highest radioactivity was selected for each mutant to be further analyzed.

Proliferation assays. The proliferation of DA-3 transfectants in response to IL-3 or Epo was determined by [^{3}H]thymidine incorporation. The cells (10^5 per well) were plated in flat-bottom 96-well plates. Following a 20-h incubation at 37°C under 5% CO_2, cells were pulsed with 1 μCi of [^{3}H]thymidine for 4 h. The cells were then harvested onto a glass-fiber filter paper, and the [^{3}H]thymidine uptake was quantitated by scintillation counting.

^{125}I-Epo binding study. Carrier-free human recombinant Epo was iodinated by using IODO-BEADS (Pierce). One bead of IODO-BEADS was incubated for 5 min with 500 μCi of ^{125}I in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.2). Epo (5 μg of protein) was then added to the reaction vial and further incubated for 4 min at room temperature. After the incubation, ^{125}I-Epo was separated from the free ^{125}I by chromatography over a Presto desalting column (Pierce). This procedure provided Epo with 0.8 atom of ^{125}I per molecule (48 μCi per μg of protein) and with full biological activity, as assayed by the ability of ^{125}I-Epo to stimulate proliferation of DA-3 cells expressing the wild-type EpoR as described above.

For the binding assay with ^{125}I-Epo, 2×10^6 cells, grown in the presence of IL-3 and the absence of Epo, in 100 μl of binding medium (RPMI 1640 medium supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 2% bovine serum albumin, and 0.02% sodium azide) were incubated with various concentrations of ^{125}I-Epo at 37°C for 90 min. After sedimentation of the cells through dibutylphthalate oil, cell-associated and free radioactivities were measured by a gamma counter. Specific binding was determined by subtracting the radioactivity associated with the parental DA-3 cells (nonspecific binding) from that associated with DA-3 transfectants (total binding). Parental DA-3 cells show no binding of Epo that can be competed by adding an excess of nonradioactive Epo and do not express the EpoR as assessed by Northern (RNA) blot analysis.

Analysis of phosphotyrosyl proteins. Radioactively labeled phosphotyrosyl proteins were affinity purified and analyzed essentially as previously described (16). In brief, cells (1×10^7 to 2×10^7) equilibrated in 1 ml of medium containing 1 mCi of $^{32}P_i$ for 3 h were either left as a control or stimulated with a saturating concentration of IL-3 (250 U/ml) or Epo (40 U/ml) unless otherwise indicated. From lysates of the cells, phosphotyrosyl proteins were affinity purified with the antiphosphotyrosine monoclonal antibody 1G2 (Oncogene Science) and analyzed by two-dimensional gel electrophoresis followed by autoradiography (16).

Immunoprecipitation analysis of the EpoR. For synthetic labeling of the EpoR, cells (5×10^6) were incubated in 0.5 ml of medium containing 200 μCi of [^{35}S]methionine for 60 min at 37°C under 5% CO_2. The cells were lysed in 500 μl of lysis buffer and immunoprecipitated with a rabbit anti-mouse EpoR antiserum as described previously (42). Immunoprecipitates were subjected to 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional gel electrophoresis followed by autoradiography.

To detect phosphorylation of the EpoR, 10^7 cells were incubated with 1 mCi of $^{32}P_i$ for 3 h in 1 ml of phosphate-free RPMI 1640 medium. Immediately before lysis, the cells were either stimulated with a saturating concentration of Epo or left untreated as a control. Lysis of the cells and immunoprecipitation of the EpoR were performed as described previously (42) except that the lysis buffer was augmented with 100 μM sodium orthovanadate.

For double immunoprecipitation, ^{32}P-labeled phosphotyrosyl proteins were isolated as described above and eluted from the antibody with 100 μl of the lysis buffer supplemented with 10 mM phenylphosphate. From this, 20 μl was used for direct analysis, 40 μl was used for immunoprecipitation with anti-EpoR antiserum, and 40 μl was used for immunoprecipitation with normal rabbit serum. After being diluted with 500 μl of the lysis buffer, the sample was subjected to immunoprecipitation analysis using the anti-EpoR antiserum.

Immunoblotting. Immunoprecipitates were resolved by SDS-PAGE and electrophoretically transferred to an Immobilon (Millipore) membrane. The blot was blocked and subsequently probed with an antiserum against the EpoR and ^{125}I-protein A.

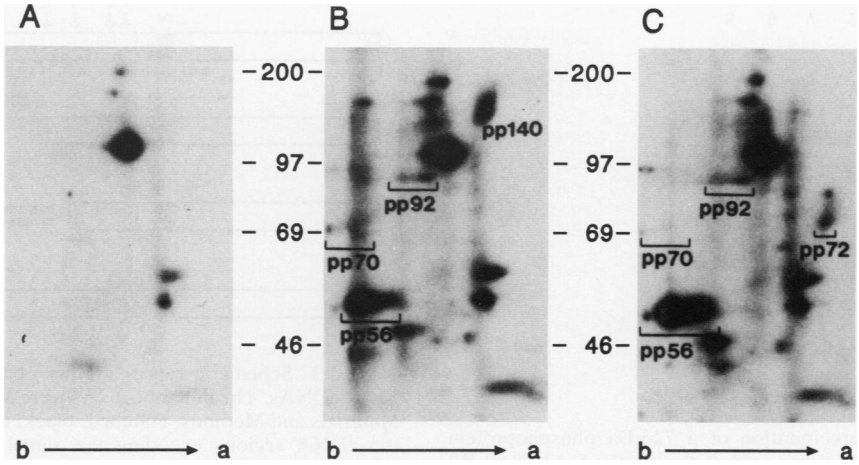

FIG. 1. Two-dimensional gel analysis of protein tyrosine phosphorylation induced by stimulation with IL-3 or Epo in DA-3 transfectants expressing the wild-type EpoR. Growth factor-depleted cells were equilibrated in $^{32}P_i$ for 3 h and left unstimulated as a control (A) or stimulated with an excess of IL-3 (B) or Epo (C) for 10 min immediately before lysis of cells. From the cell lysates, phosphotyrosine-containing proteins were isolated with antiphosphotyrosine monoclonal antibody-coupled Sepharose beads and subjected to two-dimensional analysis as described in Materials and Methods. Positions of size standards (in kilodaltons) are shown. The directions from basic (b) to acidic (a) ends of the first-dimension gel are indicated by arrows under the panels. Proteins whose tyrosine phosphorylation was induced by IL-3 or Epo are indicated with their approximate molecular sizes in panel B or C, respectively.

RESULTS

Expression of the EpoR confers Epo-dependent growth on the DA-3, IL-3-dependent myeloid cell line. To obtain Epo-dependent cell lines to investigate signal transduction mechanisms, the pXM-EpoR expression plasmid (6), containing the wild-type EpoR cDNA, was transfected with plasmid pSV2*neo* into an IL-3-dependent cell line (DA-3). The transfectants were selected for their ability to survive in the presence of G418 or Epo. Ten subclones were isolated by colony formation in soft agar and were analyzed for expression of the EpoR by ^{125}I-Epo binding as described in Materials and Methods. A subclone (DA3/EpoR-Wt) that bound the highest amount of ^{125}I-Epo was used for further studies.

Parental DA-3 cells are dependent on IL-3 and rapidly lose viability when transferred to medium with or without Epo. In contrast, DA3/EpoR-Wt cells proliferated continuously in media supplemented with 0.1 U of Epo per ml. In Epo, the cells grew comparably with respect to doubling times and saturation densities to cells grown in IL-3 or to parental cells grown in IL-3. In the absence of Epo, the DA3/EpoR-Wt cells lost viability similarly to parental cells (data not shown).

Epo and IL-3 induce similar but not identical protein tyrosine phosphorylation patterns in DA3/EpoR-Wt. To examine the ability of Epo to induce tyrosine phosphorylation, DA3/EpoR-Wt cells were cultured in $^{32}P_i$ and were stimulated with IL-3 or Epo. The phosphotyrosine-containing proteins were isolated from cell lysates by affinity purification with the antiphosphotyrosine monoclonal antibody 1G2 coupled to Sepharose (10), and the proteins were subjected to two-dimensional analysis as described in Materials and Methods. IL-3 stimulation induced the appearance of several predominant proteins, including pp140, pp92, and pp56 (Fig. 1B). In addition, a minor phosphoprotein of 70 kDa was more variably detected. This pattern is similar to that obtained with the parental DA-3 cells (data not shown) as well as other IL-3-dependent cell lines (16). Epo stimulation resulted in the appearance of several similar proteins, including pp92, pp70, and pp56 (Fig. 1C). However, pp140, which has been hypothesized to be a component of the IL-3 receptor complex (18), was not observed. In addition, Epo uniquely induced the phosphorylation of a protein of approximately 72 kDa. Induction of phosphorylation was observed with concentrations of Epo as low as 0.1 U/ml and was maximal at a concentration of 4 U/ml (data not shown). This concentration range is similar to the dose response for Epo in proliferation assays.

The kinetics of appearance of the phosphoproteins were also examined. Phosphorylation was detectable as early as 1 min after Epo addition, peaked at 3 min, and persisted for at least 30 min. After 60 min, the pattern returned to that of unstimulated cells (data not shown). These kinetics are comparable to those observed following IL-3 stimulation of various IL-3-dependent cell lines (16).

Phosphorylation of the EpoR. The size of the Epo-induced 72-kDa phosphoprotein suggested that it may be the EpoR. To explore this possibility, we immunoprecipitated 1G2 eluates from control and Epo-stimulated cells. As shown in Fig. 2A, EpoR antisera immunoprecipitated a 72-kDa phosphoprotein (lane 6). For comparison, lane 3 contains the total eluate from 1G2. The 72-kDa phosphoprotein was not seen in unstimulated cells (lane 5) or with control sera (lane 9). However, in these experiments it was noted that the 72-kDa phosphoprotein migrated slower than the reported (42) EpoR forms, which include a minor 62-kDa nonglycosylated form, a 64-kDa glycosylated form which is endoglycosidase H sensitive, and a further processed form of 66 kDa which has complex, endoglycosidase H-resistant carbohydrate (indicated by the asterisk in Fig. 2A).

The results of these experiments were consistent with either (i) the phosphorylation of a small fraction of induction of tyrosine EpoR the following Epo stimulation or (ii) the association of EpoR with a phosphoprotein of 72 kDa which is tyrosine phosphorylated following Epo stimulation. To distinguish these possibilities, Western immunoblotting experiments were done. DA3/EpoR-Wt cells were stimulated with Epo, and the phosphotyrosine-containing proteins were

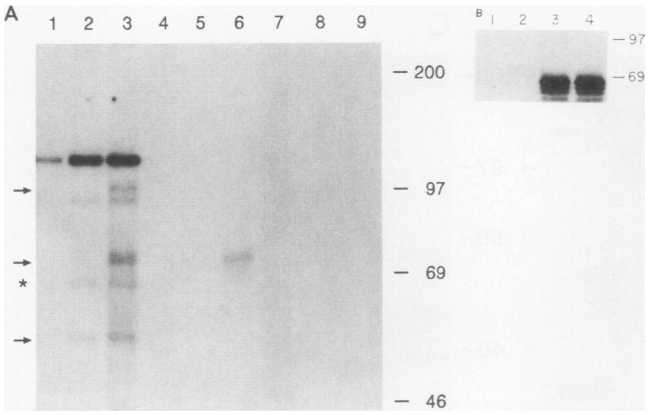

FIG. 2. (A) Immunoprecipitation of a 72-kDa phosphoprotein from eluates of 1G2 by antisera against the EpoR. DA3/EpoR-Wt cells were labeled with $^{32}P_i$ and either stimulated with Epo for 10 min (lanes 3, 6, and 9) or left unstimulated (lanes 2, 5, and 8). As a control, parental DA-3 cells were labeled under comparable conditions (lanes 1, 4, and 7). Phosphotyrosine-containing proteins were then purified by 1G2 affinity beads as described in Materials and Methods. The phosphoprotein fraction was either directly subjected to SDS-PAGE (lanes 1 to 3) or used for immunoprecipitation with antisera against the EpoR (lanes 4 to 6) or normal rabbit serum (lanes 7 to 9), and the immunoprecipitates were subjected to SDS-PAGE. Arrows indicate the positions of migration of the phosphoproteins substrates seen following Epo stimulation; the asterisk indicates the position of migration of methionine-labeled, immunoprecipitated EpoR from other experiments. (B) Anti-EpoR immunoblotting of the proteins isolated with a monoclonal antibody against phosphotyrosine. DA3/EpoR-Wt cells (5×10^7) were either left unstimulated as a control (lanes 1 and 3) or stimulated with Epo (lanes 2 and 4). Phosphotyrosine-containing proteins were isolated by immunoprecipitation with 1G2 (lanes 1 and 2), or the EpoR was immunoprecipitated with an antiserum against EpoR from the supernatants of 1G2 (lanes 3 and 4). In each case, the proteins were separated by SDS-PAGE and blotted onto Immobilon membranes. The blot was probed with the EpoR antiserum and ^{125}I-protein A. Positions of size markers (in kilodaltons) are shown.

isolated with 1G2. Both the eluates and the EpoR immunoprecipitated from the non-1G2-binding fractions were resolved by SDS-PAGE, blotted to Immobilon, and probed with EpoR antisera. As shown in Fig. 2B, a very minor 72-kDa protein was specifically seen in eluates from Epo-stimulated cells. This protein was consistently seen at low levels in several experiments. In contrast, the major immunoreactive material was found in the protein fraction that did not bind to 1G2 and migrated with the expected size of EpoR. These results are most consistent with the tyrosine phosphorylation of a small fraction of the EpoR which affects its migration in SDS-PAGE.

Correlation between Epo-induced protein tyrosine phosphorylation and mitogenesis. To determine the role of protein tyrosine phosphorylation in growth regulation by Epo, we constructed and expressed deletion mutants of the EpoR. The mutants were then analyzed for their ability to support growth or to induce tyrosine phosphorylation. The structures of three mutants are shown in Fig. 3. Two mutants contained carboxyl truncations resulting in deletions of 108 and 146 amino acids (H-mutant and S-mutant, respectively). A third mutant (PB-mutant) contains an internal deletion of 20 amino acids. This deletion removes a region that has sequence homology to a portion of the IL-2 receptor β chain that has been shown to be critical for transducing an IL-2-

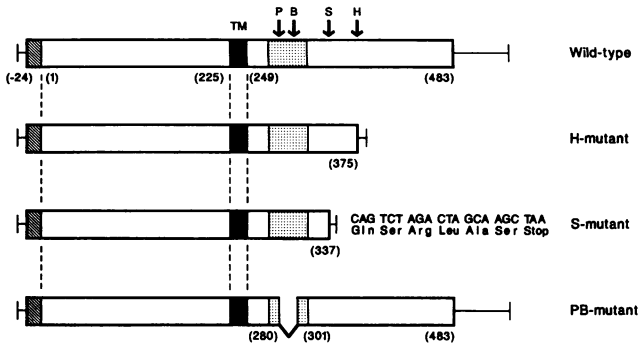

FIG. 3. Schematic representation of the wild-type and mutant EpoR cDNAs. The mutant cDNAs were constructed as described in Materials and Methods. Hatched, black, and dotted areas represent the cDNA regions encoding the signal peptide, transmembrane domain, and an intracellular region homologous to a functionally critical region of the IL-2 receptor β chain, respectively. Numbers in parentheses correspond to amino acid residues. As indicated in the figure and in Materials and Methods, six amino acids were introduced to the carboxyl end of the S-mutant because of a base deletion in the linker that was used to introduce stop codons. Relevant restriction enzyme sites are indicated at the top: P, *Pvu*II; B, *Bam*HI; S, *Sph*I; H, *Hin*dIII.

induced mitogenic signal (13). The mutant cDNAs were inserted into the pXM expression vector and transfected into DA-3 cells. From the transfectants, several single cell clones expressing high levels of the receptors were selected. The results obtained with three of the clones, designated DA3/EpoR-H, DA3/EpoR-S, and DA3/EpoR-PB, are shown.

The clones were first analyzed for expression of the mutant receptors by immunoprecipitation. Clones were metabolically labeled with [^{35}S]methionine, and cell extracts were immunoprecipitated with antisera against the EpoR. As shown in Fig. 4, individual clones expressed proteins of 47 kDa (H-mutant), 42 kDa (S-mutant), and 64 kDa (PB-mutant) which are consistent with the sizes of the deletions. We next examined the cells for the affinity and levels of expression of ^{125}I-Epo binding activity. Scatchard plots of the binding data are shown in Fig. 5. All of the clones exhibited a single class

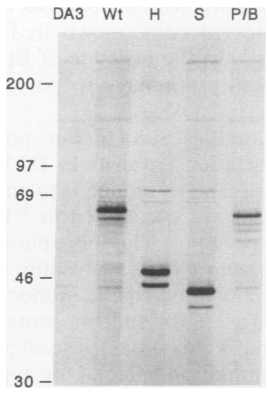

FIG. 4. Expression of the wild-type and mutant EpoRs in DA-3 transfectants. From cells metabolically labeled with [^{35}S]methionine, the EpoRs were immunoprecipitated with an anti-EpoR serum and subjected to SDS-PAGE followed by autoradiography. Lanes: DA-3, parental DA-3; Wt, DA3/EpoR-Wt; H, DA3/EpoR-H; S, DA3/EpoR-S; P/B, DA3/EpoR-PB.

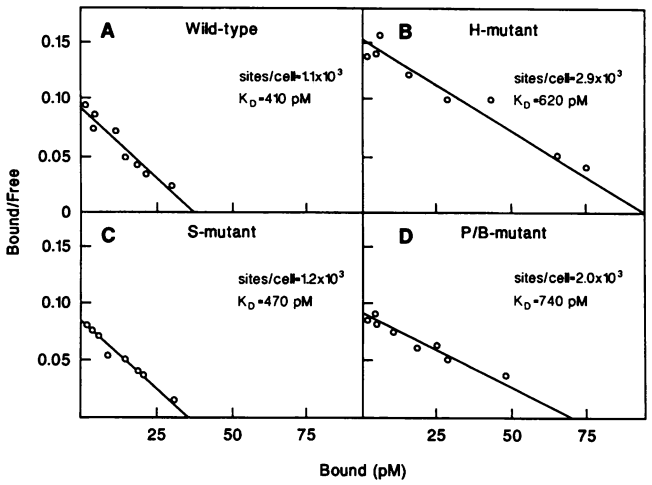

FIG. 5. Scatchard plot analysis of ^{125}I-Epo binding to DA-3 transfectants. ^{125}I-Epo binding to DA3/EpoR-Wt (A), DA3/EpoR-H (B), DA3/EpoR-S (C), and DA3/EpoR-PB (D) was determined as described in Materials and Methods. Binding studies were performed to saturation by using increasing concentrations of ^{125}I-Epo. The specific binding at each concentration was determined by subtraction of the radioactivity associated with the parental DA-3 cells (nonspecific binding) from that associated with DA-3 transfectants (total binding). The binding data were then plotted and analyzed by the method of Scatchard.

of binding sites with an apparent affinity of 400 to 800 pM. Moreover, each mutant clone expressed levels of receptors comparable to those of cells expressing the wild-type receptor.

The mitogenic response of the various clones was examined by [^{3}H]thymidine incorporation assays (Fig. 6). All of the cell clones responded comparably to IL-3 (data not shown). DA3/EpoR-Wt cells, expressing the wild-type receptor, showed a maximal mitogenic response to Epo that was comparable to that obtained with IL-3. Cells (DA3/EpoR-H) expressing the H-mutant responded to only approximately one-third of the response to IL-3. Cells (DA3/EpoR-S) expressing the S-mutant responded to only 3% of the level obtained with IL-3. Strikingly, cells containing the internal 20-amino-acid deletion (DA3/EpoR-PB) had no significant response to Epo, suggesting that this region is essential for mitogenesis.

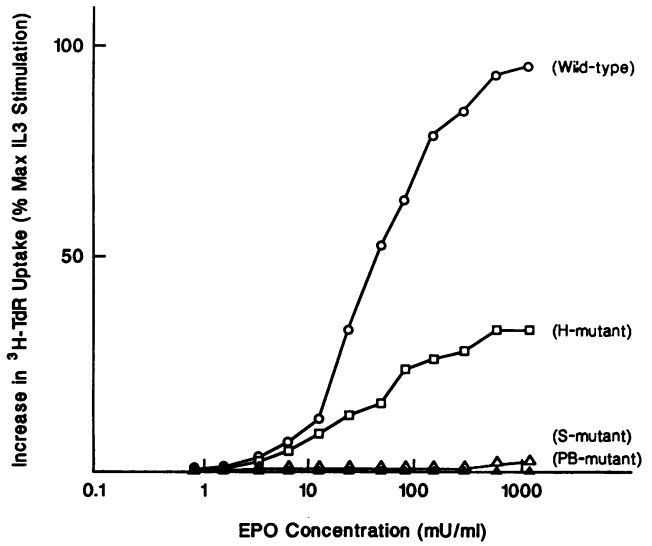

FIG. 6. Comparison of mitogenic response of DA-3 transfectants to Epo. DNA synthesis by DA3/EpoR-Wt (○), DA3/EpoR-H (□), DA3/EpoR-S (△), and DA3/EpoR-PB (▲) was measured by [^{3}H]thymidine incorporation as described in Materials and Methods. Increase in [^{3}H]thymidine incorporation induced by increasing concentrations of Epo is expressed as a percentage of that induced by the maximal stimulatory level of IL-3 (25 U/ml).

The clones expressing mutant forms of the EpoR were next examined for their ability to induce tyrosine phosphorylation. As illustrated in Fig. 7C, Epo treatment of DA3/EpoR-H cells induced the appearance of phosphoproteins of 92, 70, and 56 kDa, similar to the pattern seen with cells stimulated with IL-3. Phosphorylation of the 72-kDa substrate was not observed in repeated experiments. However, in all of the experiments, the extent of induction of phosphorylation was consistently less than that observed with DA3/EpoR-H cells stimulated with IL-3. In contrast, Epo failed to induce the typical pattern of tyrosine phosphoproteins in DA3/EpoR-S or DA3/EpoR-PB cells stimulated with Epo in repeated experiments (Fig. 8C). Thus, the abilities of these receptors to induce tyrosine phosphorylation correlated with the ability to transduce a mitogenic signal.

DISCUSSION

A variety of studies have implicated tyrosine phosphorylation in the growth regulation of hematopoietic cells. Initially it was demonstrated that various oncogenes that encode tyrosine kinases could abrogate the requirements of myeloid cell lines for IL-3 (5, 30). More recently, temperature-sensitive mutants of Abelson murine leukemia virus have been shown to confer a temperature-dependent phenotype for IL-3 dependence for growth (21) and for the transcriptional regulation of immediate-early genes (4). Second, introduction of various growth factor receptors containing tyrosine protein kinase domains confers on IL-3-dependent myeloid cells the ability to proliferate in response to their ligands (20, 31, 33). Lastly, IL-3 has been shown to induce the rapid tyrosine phosphorylation of a series of substrates (16, 22, 29), including an IL-3-binding protein of 140 kDa (18). Our studies further implicate tyrosine phosphorylation in growth regulation by demonstrating that the EpoR induces tyrosine phosphorylation and that, in mutants, this ability is correlated with mitogenesis.

Expression of the wild-type EpoR in IL-3-dependent cells allowed the cells to proliferate in response to Epo in a manner that was comparable to their response to IL-3. Of particular note was the observation that although many of the clones have been grown for extended periods of time in Epo, there has been no evidence for differentiation along the erythroid lineage, including the expression of β-globin or band 3 (23). This response is different from the responses of two other IL-3-dependent cell lines, 32Dc13 (26) and B6SUtA (2, 9), which differentiate in response to Epo. Since these cells express the endogenous EpoR, it is conceivable that they are at a point in differentiation at which Epo stimulation can also induce differentiation. In contrast, DA-3 cells may represent myeloid cells at an earlier stage of differentiation or cells that are committed to other myeloid lineages. Irrespective, the results demonstrate that the ability of the EpoR to support proliferation can be separated from its potential effects on differentiation.

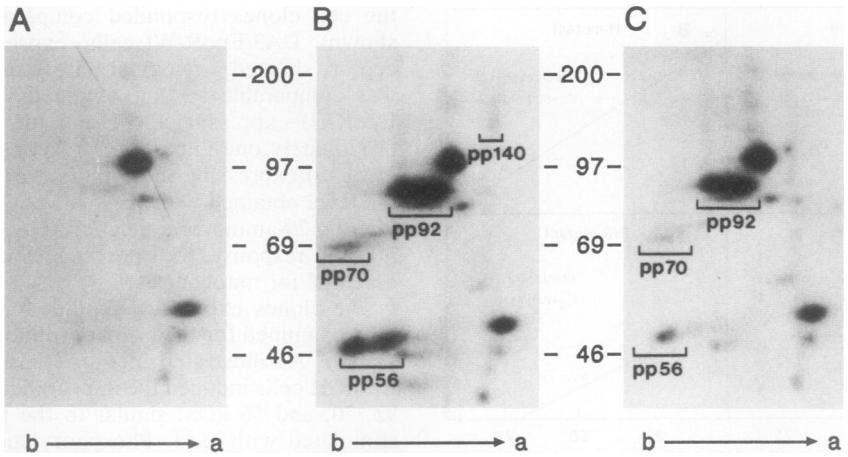

FIG. 7. Two-dimensional gel analysis of protein tyrosine phosphorylation in DA-3 transfectants expressing H-mutant EpoR. DA3/EpoR-H cells were equilibrated in $^{32}P_i$ for 3 h and then either not stimulated (A) or stimulated with an excess of IL-3 (B) or Epo (C). Phosphotyrosyl proteins were purified and analyzed by two-dimensional electrophoresis and autoradiography as described in Materials and Methods. Positions of size standards (in kilodaltons) are shown. The directions from basic (b) to acidic (a) ends of the first-dimension gel are indicated by arrows under the panels. The positions and approximate molecular sizes of phosphoproteins corresponding to those indicated in Fig. 1 are indicated.

Epo stimulation of cells transfected with the wild-type receptor induces rapid tyrosine phosphorylation of a distinct set of substrates. These results support recent studies (32) in which Epo was shown to induce tyrosine phosphorylation in the Epo-responsive cell line B6SUt.EP. In these cells, substrates of 153, 140, 100, 93, 74, and 54 kDa were observed. It is possible that the 93-, 74-, and 54-kDa proteins are the same that we have observed. We have not detected substrates of 153, 140, or 100 kDa in our studies. This could be due to differences in the cell lines, possibly related to the differences in the ability to differentiate or to the reagents used to detect tyrosine-phosphorylated proteins. Our studies have relied on a monoclonal antibody (1G2) for affinity purification, while the experiments of Quelle and Wojchowski (32) utilized a polyvalent antiserum in Western blotting.

The identities of the substrates of phosphorylation that are seen with both IL-3 and Epo in DA-3 cells are not known, although several possibilities have been examined. In particular, the 92-kDa substrate is not immunoprecipitated by two different antisera against the *fes* tyrosine protein kinase. The possibility that the 56-kDa substrate is a member of the *src* family of kinases has been examined. By Northern analysis or by PCR amplification of conserved kinase domains, the cells used in these studies (DA-3) do not express *src*, *lck*, or *hck* but do express *lyn*. However, an antiserum against *lyn* does not immunoprecipitate p56 (40). Lastly, in one study it was shown that the 74-kDa c-*raf* kinase is a

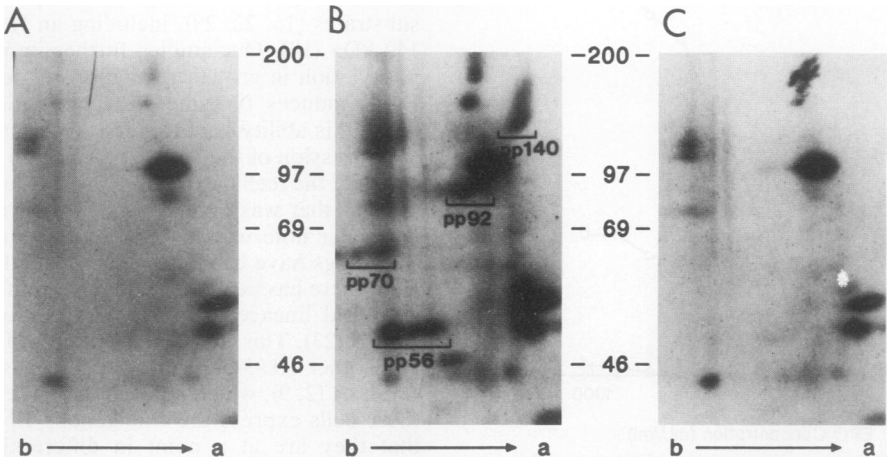

FIG. 8. Two-dimensional gel analysis of protein tyrosine phosphorylation in DA-3 transfectants expressing PB-mutant EpoR. DA3/EpoR-PB cells were equilibrated in $^{32}P_i$ for 3 h and then either not stimulated (A) or stimulated with an excess of IL-3 (B) or Epo (C). Phosphotyrosyl proteins were purified and analyzed by two-dimensional electrophoresis and autoradiography as described in Materials and Methods. Positions of size standards (in kilodaltons) are shown. The directions from basic (b) to acidic (a) ends of the first-dimension gel are indicated by arrows under the panels. The positions and approximate molecular sizes of phosphoproteins corresponding to those indicated in Fig. 1 are indicated.

substrate for tyrosine phosphorylation in IL-3-dependent cells (3). In a second study, however, c-*raf* was not significantly phosphorylated on tyrosine (19). The 70-kDa substrate that we have observed following IL-3 stimulation is not immunoprecipitated by antisera against c-*raf* (17). Therefore, it will be important to purify these substrates to clone the genes and ultimately determine their potential role in signal transduction.

Since phosphorylation of membrane receptors plays a critical role in the regulation of receptor function and distribution, we examined the phosphorylation status of the EpoR in transfected DA-3 cells. The EpoR is not heavily phosphorylated, as determined by the lack of comigration of a major $^{32}P_i$ label with the methionine-labeled EpoR (data not shown). However, immunoprecipitation and immunoblotting studies using an antiserum against the EpoR indicated that the Epo-specific, 72-kDa substrate of tyrosine phosphorylation is the EpoR itself. The induction of tyrosine phosphorylation of the EpoR is compatible with the hypothesis that the receptor transduces its signal by coupling with tyrosine kinases. However, the low stoichiometry of phosphorylation suggests that the phosphorylation of the EpoR is unlikely to play a critical role in signal transduction or receptor function.

Importantly, our studies demonstrate a correlation between the ability of Epo binding to support growth and to induce tyrosine phosphorylation. Of particular note was the H-mutant, in which there was a proportional loss between mitogenic activity and phosphorylation. For the PB mutant, removal of 20 amino acids completely eliminated both activities. Thus, we conclude that tyrosine phosphorylation is involved in Epo-induced mitogenesis. Our results also support previous studies (7) which have demonstrated that a membrane-proximal region of the EpoR is required for signal transduction. However, in contrast to these studies, we have not observed a significant enhancement of mitogenic activity by carboxyl truncations. This may be due to the differences in the lineages of cells that were used.

A critical domain for mitogenesis and for induction of phosphorylation was identified in our studies and lies approximately 35 amino acids from the transmembrane domain. This region may be critical for appropriate folding of the cytoplasmic domain or may be a region required for interaction with other signal-transducing proteins. The latter is suggested by the relatively small effect that is seen by carboxyl truncations of 108 amino acids. Importantly, the critical EpoR domain is within the region that exhibits sequence homology with the IL-2 β receptor and which has been shown to be essential for the mitogenic activity of the IL-2 receptor when expressed in IL-3-dependent cells (12). With the IL-2 β receptor, it had been speculated that it was the serine richness of this region that may be important. However, the corresponding region of the EpoR is not particularly serine rich; thus, the sequence may be more important, in particular those sequences that are shared between the EpoR and the IL-2 β receptor. This question can be further addressed by examining point mutations within this region.

An important result of our studies suggests that the receptor must couple through a cellular kinase to bring about the phosphorylations that are observed. This coupling might involve a physical association between the receptor and a kinase similar to the association of the *lck* kinase with CD4 or CD8 (37–39) and *fyn* with the T-cell receptor (35). For this reason, we have examined immunoprecipitates of the EpoR for kinase activity and have failed to detect any (41). This may simply reflect a lower affinity of interaction. Because of the sequence similarity of the IL-2 β receptor and the EpoR and the ability of IL-2 to activate $p56^{lck}$ (14), it is intriguing to speculate the EpoR may associate with the *lck* kinase. However, DA-3 cells do not express *lck* (40). Irrespective, our results further implicate tyrosine phosphorylation in hematopoietic growth factor signal transduction.

ACKNOWLEDGMENTS

This work was supported in part by National Cancer Institute Cancer Center Support (CORE) grant P30 CA21765, by grant RO1 DK42932 from the National Institute of Diabetes and Digestive and Kidney Diseases, by grant CA25810 from the National Cancer Institute, and by the American Lebanese Syrian Associated Charities. A.D.D. is a Lucille P. Markey Scholar, and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust.

We thank Susan Kozak for excellent assistance.

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