MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1788–1795
0270-7306/93/031788-08$02.00/0

Vol. 13, No. 3

Inactivation of Erythropoietin Receptor Function by Point Mutations in a Region Having Homology with Other Cytokine Receptors

OSAMU MIURA,[1]† JOHN L. CLEVELAND,[1] AND JAMES N. IHLE[1,2]*

Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 31051,[1] and Department of Biochemistry, University of Tennessee, Memphis, Tennessee 381633[2]

Received 13 July 1992/Returned for modification 12 August 1992/Accepted 21 December 1992

The cytoplasmic domain of the erythropoietin receptor (EpoR) contains a region, proximal to the transmembrane domain, that is essential for function and has homology with other members of the cytokine receptor family. To explore the functional significance of this region and to identify critical residues, we introduced several amino acid substitutions and examined their effects on erythropoietin-induced mitogenesis, tyrosine phosphorylation, and expression of immediate-early (c-*fos*, c-*myc*, and *egr*-1) and early (ornithine decarboxylase and T-cell receptor γ) genes in interleukin-3-dependent cell lines. Amino acid substitution of W-282, which is strictly conserved at the middle portion of the homology region, completely abolished all the functions of the EpoR. Point mutation at L-306 or E-307, both of which are in a conserved LEVL motif, drastically impaired the function of the receptor in all assays. Other point mutations, introduced into less conserved amino acid residues, did not significantly impair the function of the receptor. These results demonstrate that conserved amino acid residues in this domain of the EpoR are required for mitogenesis, stimulation of tyrosine phosphorylation, and induction of immediate-early and early genes.

The growth and differentiation of hematopoietic cells are regulated by various cytokines which act through specific receptors (3, 22). Most of the hematopoietic growth factor receptors, including the receptor for erythropoietin (EpoR), belong to a cytokine receptor family (2, 6). Members of this family characteristically have four conserved cysteine residues and the WSXWS motif in the extracellular domain. The WSXWS motif has been suggested to be critical for the folding of the extracellular domain of the interleukin-2 receptor β subunit (IL-2βR) (26). In contrast, the intracellular domains of these receptors are quite different and do not contain motifs that would indicate potential functions in signal transduction. Recently, however, limited sequence similarity has been noted within the cytoplasmic domains of several of the hematopoietic growth factor receptors (11, 26, 27). The functional significance of these regions has not been established.

The signal-transducing events that are activated by stimulation of hematopoietic growth factor receptors have been extensively studied and have provided evidence for a role for tyrosine phosphorylation in growth regulation. In particular, constitutively activated tyrosine kinases abrogate the requirement of hematopoietic cells for growth factors (21, 28), and temperature-sensitive mutants confer a conditional requirement for growth factors (4, 18). A number of hematopoietic growth factors, including Epo, have been shown to rapidly induce tyrosine phosphorylation (24, 30). Strikingly, different growth factors, such as Epo and interleukin-3 (IL-3), induce the phosphorylation of a comparable set of substrates (24). A correlation has been shown between mitogenesis and the ability to induce tyrosine phosphorylation among a series of carboxyl and internal deletions of EpoR (24).

Although hematopoietic growth factor receptors induce tyrosine phosphorylation, little is known about the tyrosine kinases that may associate with the receptors and be involved in mitogenesis. The simplest hypothesis is that a tyrosine kinase physically associates with members of the cytokine receptor family. The similar response that is induced by various growth factors has been interpreted to indicate that common, or highly related, tyrosine kinases associate with different receptors. Recently several studies have shown an association of *lck* with IL-2βR (13) or activation of *lck* kinase activity following IL-2 binding (16). However, *lck* associates with a region of the receptor that is not required for a mitogenic response (13), and IL-2βR induces mitogenesis in cells that do not express *lck*. Therefore, it is likely that other domains within the receptor may associate with different tyrosine kinases to initiate a mitogenic response.

Several studies have begun to define the domains of hematopoietic growth factors that are required for mitogenesis. These studies have shown that the carboxyl half of the cytoplasmic domain is not required for a mitogenic response for the interleukin-6 receptor (IL-6R)-associated gp130 (27), granulocyte colony-stimulating factor (G-CSF) receptor (11), IL-2βR (14), or EpoR (8, 24, 29). However, in Ba/F3 cells, the cytoplasmic tail of the EpoR can inhibit the mitogenic response (8), and in FDC-P1 cells, it down-modulates the action of the granulocyte-macrophage colony-stimulating factor receptor (29). Internal deletions and point mutations have begun to focus attention on a region of the cytoplasmic domain that shows limited sequence identity among a number of the hematopoietic growth factor receptors (11, 26, 27). This region is of particular interest since many of the receptors mediate comparable events in cells and are thus speculated to associate with common signal-transducing proteins. Consistent with this view, some chimeric receptors have been shown to retain the ability to induce mitogenesis (37). Deletion of a 20-amino-acid or a 46-amino-acid segment

* Corresponding author.
† Present address: Department of First Internal Medicine, Tokyo Medical and Dental University, Tokyo, Japan.

within the conserved region in the EpoR (24) or IL-2βR (14), respectively, inactivates these receptors. Moreover mutations in conserved sequences in this region of the IL-2βR (26) or the IL-6R-associated gp130 (27) can inactivate receptor function. To extend these studies, we have constructed a series of mutants of the EpoR containing point mutations in conserved amino acids and demonstrate that these changes can inactivate receptor function, as assessed by the ability to induce mitogenesis, to stimulate tyrosine phosphorylation, and to induce immediate-early and early response genes.

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 (17), were maintained as described previously (24). DA3/EpoR-Wt, a clone of DA-3 cells expressing the EpoR obtained by transfecting the wild-type (wt) EpoR cDNA, has been previously described (24). A clone of 32Dcl cells that has been previously described (23) was used in these studies. An expression plasmid for the murine EpoR, pXM-EpoR, and a rabbit polyclonal antipeptide antiserum directed against the amino terminus of the murine EpoR have also been previously described (7, 35). Recombinant human Epo was kindly provided by Amgen Biological (Thousand Oaks, Calif.). All other reagents were purchased from commercial sources unless otherwise noted.

Construction of expression plasmids for EpoR mutants and transfection into DA-3 cells. Site-directed mutagenesis of amino acids within the region of the EpoR showing homology with the IL-2β receptor chain was carried out by primer-mediated mutagenesis using the polymerase chain reaction (PCR) method as described by Higuchi (15). In brief, two complementary primers having a mutation to be introduced were synthesized and used in combination with one of two primers on either side of the cloning site of the pXM vector for the first-step PCR. After purification from agarose gels, the two PCR products were mixed and subjected to the second-step PCR, using the two primers encompassing the cloning site. The final product was then subcloned into the pXM vector, and the presence of the introduced mutation as well as the integrity of the resulting plasmid was confirmed by DNA sequencing using primers within the EpoR cDNA and by digestion with restriction enzymes. Constructed expression plasmids were transfected into DA-3 and 32Dcl cells with the pSV2*neo* plasmid by the electroporation method, and clones were selected for the resistance to G418 as described previously (24). Clones that expressed the most cell surface EpoR were then selected by binding assays with ^{125}I-Epo as described previously (24) and subjected to the following studies.

Analysis of the mutant EpoRs by ^{125}I-Epo binding and immunoprecipitation studies. Iodination of Epo by using IODO-BEADS (Pierce) and binding assay of the receptors with ^{125}I-Epo for the Scatchard plot were carried out as described previously (24). Synthetic labeling of the EpoR and immunoprecipitation of the receptor with a rabbit anti-murine EpoR antiserum were carried out as described previously (24) except that a mixture of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-Label; ICN), instead of pure [^{35}S]methionine, was used to label cells for 2 h.

Proliferation assays. The proliferation of DA-3 and 32Dcl transfectants in response to IL-3 or Epo was determined by [^{3}H]thymidine incorporation as described previously (24).

Immunoblotting. For immunoblot analysis with an antiphosphotyrosine monoclonal antibody, 4G10 (U.S. Biochemical), cells were washed free of IL-3, cultured overnight, and left unstimulated as a control or stimulated with a saturating concentration of Epo or IL-3. Cells were then lysed in the lysis buffer previously described for analysis of phosphotyrosyl proteins by immunoprecipitation (24). After clarification by spinning at 15,000 rpm for 20 min in a refrigerated microcentrifuge, lysates were mixed with equal volumes of 2× Laemmli's sodium dodecyl sulfate (SDS) sample buffer and heated at 100°C for 5 min. A sample from 4×10^5 cells was applied to each well of SDS–8.5% polyacrylamide gels, electrophoresed, and blotted onto nitrocellulose membranes. Membranes were probed with the 4G10 antibody followed by detection with an ECL Western immunoblotting detection system (Amersham).

To examine the tyrosine phosphorylation of the EpoR, the receptors were first purified by immunoprecipitation with a rabbit antiserum raised against a bacterial recombinant protein containing the cytoplasmic region of the EpoR. The receptors were then electrophoresed on SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were first probed with the 4G10 antibody as described above. The filters were then treated with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) at 50°C for 30 min and were subsequently probed with the antiserum against the recombinant EpoR.

Isolation and analysis of RNA. Cells were washed free of IL-3, cultured for 24 h, and then stimulated with IL-3 or Epo for various periods. Total RNA was extracted from cells and analyzed by Northern (RNA) blotting as described previously (4). The DNA probes used for analyses were a 950-bp *Xba*I-*Sst*I mouse c-*myc* exon 2 fragment, a 1.6-kb *Bam*HI-*Eco*RI mouse ornithine decarboxylase (ODC) cDNA fragment, and a 1.8-kb *Pst*I fragment of the chicken β-actin cDNA. The probe for *egr*-1 was a 1.4-kb *Eco*RI fragment of the murine cDNA. The probe for the nonrearranged γ T-cell receptor locus (TCRγ) was a *Pst*I 600-bp murine genomic fragment that detects the constant region of TCRγ1-3 (34).

RESULTS

Site-directed mutagenesis of EpoR and expression in IL-3-dependent myeloid cells. We have previously identified regions of the EpoR that are critical for mitogenic activity by carboxyl and internal deletions (24). Of particular interest was an internal deletion of 20 amino acids (L-281 to E-300) in the EpoR-PB mutant which completely inactivated the receptor for mitogenesis. As shown in Fig. 1B, this region shows similarity to a region of the IL-2β receptor that had also been shown to be required for an IL-2 mitogenic response (14). Also shown for comparison are the sequences for several other hematopoietic growth factor receptors. If this region is important for receptor function, point mutations would be anticipated to affect function and might allow the identification of critical residues. Since essential residues would be anticipated to be conserved, we explored the effects of changing the most highly conserved residues. Of particular interest were (i) the sequence Q-280–L-281–W-282–L-283, which contains the W-282 residue that is conserved in several hematopoietic growth factors (27), (ii) the conserved sequence S-293–P-294, (iii) the conserved sequence P-299–E-300, and (iv) the sequence L-306–E-307–V-308–L-309, which contains the highly conserved L and E residues. To assess these regions, the indicated mutations were introduced and the mutant EpoR cDNAs were inserted into the pXM expression plasmid. The resulting constructs were cotransfected with pSV2*neo* into an IL-3-dependent

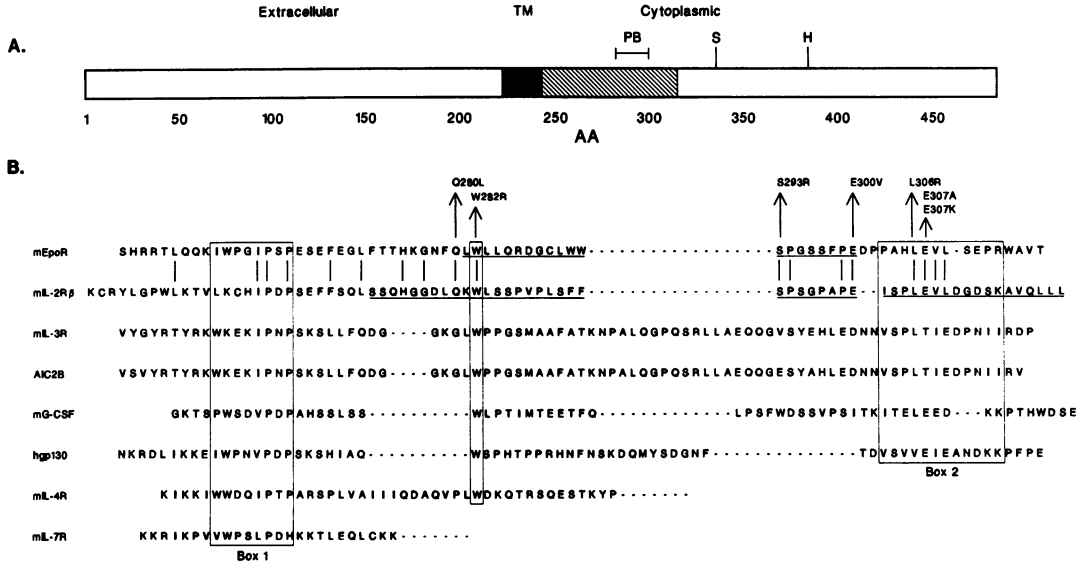

FIG. 1. Schematic structure of the EpoR and point mutations introduced into the cytoplasmic domain of the EpoR that has homology to the IL-2 receptor chain. (A) Schematic representation of the EpoR. A black box shows the transmembrane domain (TM) of the receptor. A hatched box indicates the cytoplasmic region that shows homology with the IL-2β receptor chain and various other receptors belonging to the cytokine receptor family. AA, Amino acids. (B) Comparison of the sequences of various hematopoietic growth factor receptors and positions of the point mutations introduced by site-directed mutagenesis. Sequence alignment between the murine EpoR (7) and the murine IL-2β receptor chain (19) within the hatched area of the EpoR shown in panel A. A match of the amino acid residue between the murine EpoR (mEpoR) and the human IL-2 receptor chain is indicated by . For comparison, the sequences of the comparable regions of the murine IL-3R (mIL-3R, AIC2B), the murine G-CSF receptor, the murine receptors for IL-4 and IL-7, and the IL-6R-associated 130-kDa glycoprotein (hgp130) are shown. Previously defined regions of homology, termed homology boxes 1 and 2, are shown (27). The underlined amino acids were deleted in the mitogenically detected EpoR-PR mutant (24) or in a mitogenically inactive mutant of the IL-2βR (14).

cell line, DA-3. Transfected clones were selected for resistance to G418, and individual clones that bound the highest levels of ^{125}I-Epo were selected for further studies. The clones are designated according to the point mutations that they express, e.g., DA3/EpoR-Q280L.

Expression of the mutant receptors was first examined by immunoprecipitation from metabolically labeled cells. As shown in Fig. 2, the wt EpoR appeared as a mature, 66-kDa form and smaller immature or unglycosylated forms, consistent with previous reports (24, 35). All of the mutant receptors were expressed at levels comparable to that of the wt EpoR. The three receptors with point mutations in the amino-terminal region migrated comparably to the wt EpoR, while the four receptors with mutations in the carboxyl-terminal region migrated slightly faster than the wt EpoR. The number and affinity of the Epo binding sites were analyzed by Scatchard plots of binding data (Table 1). All of the clones exhibited a single class of binding sites with an apparent affinity of 700 to 1,200 pM. Most of the clones expressed from 400 to 1,200 binding sites per cell, while the clones expressing the E300V and E307A mutations expressed 70 and 240 binding sites per cell, respectively.

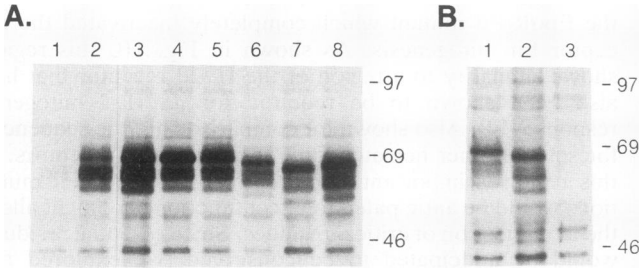

FIG. 2. Expression of the wt and mutant EpoRs in DA-3 transfectants. Parental DA-3 cells and those expressing wild-type or mutant EpoR were metabolically labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine, and the EpoR was immunoprecipitated with an antiserum against the amino terminus of the EpoR. Immunoprecipitated receptors were subjected to SDS–8.5% PAGE followed by fluorography. Positions of size standards (in kilodaltons) are shown at the right. (A) Lanes: 1, parental DA-3; 2, DA3/EpoR-Wt; 3, DA3/EpoR-Q280L; 4, DA3/EpoR-W282R; 5, DA3/EpoR-S293R; 6, DA3/EpoR-E300V; 7, DA3/EpoR-E307K; 8, DA3/EpoR-E307A. (B) Lanes: 1, DA3/EpoR-Wt; 2, DA3/EpoR-L306R; 3, parental DA-3.

Functional analysis of EpoRs containing point mutations. Expression of the wt EpoR in DA-3 cells confers Epo-dependent growth without inducing erythroid differentiation (24). The ability of Epo to induce [^{3}H]thymidine incorporation in clones of cells expressing EpoR mutants is shown in Fig. 3; the results are standardized to the maximal response

TABLE 1. Number and affinity of mutant EpoRs expressed in DA3 transfectant clones

Construct	No. of sites/cell	K_D (pM)
wt	1,100	410
Q280L	430	900
W282R	770	1,200
S293R	820	1,100
E300V	70	780
L306R	770	760
E307K	240	700
E307A	1,200	1,100

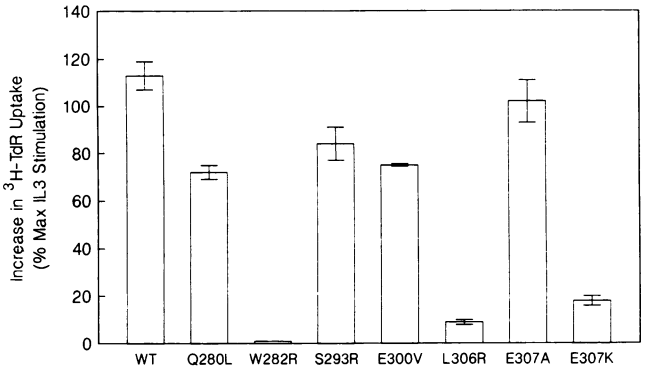

FIG. 3. Transduction of the growth signal through mutant EpoRs. DNA synthesis by DA-3 cells expressing the wt EpoR or various mutant EpoRs with point mutations, as designated under the *x* axis, was measured by the [^{3}H]thymidine (^{3}H-TdR) incorporation assay as described in Materials and Methods. Increase in [^{3}H]thymidine uptake induced by Epo stimulation is expressed as a percentage of that induced by the maximal stimulatory level of IL-3 (25 U/ml). Measurements were done in triplicate, and standard deviations are shown by vertical lines.

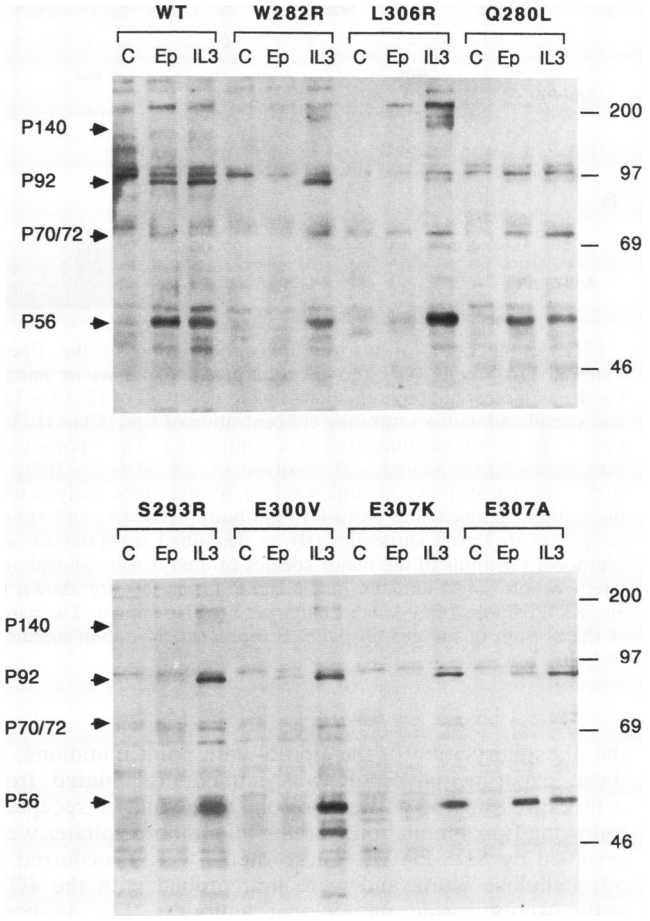

FIG. 4. Induction of protein tyrosine phosphorylation by mutant EpoRs. DA-3 cells expressing the wt EpoR or various mutant EpoRs, as designated above the lanes, were depleted of IL-3 for 12 h and left unstimulated as a control (C) or stimulated with Epo (Ep) or IL-3 (IL3). Cell lysates were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and immunoblotted with an antiphosphotyrosine monoclonal antibody as described in Materials and Methods. Phosphotyrosyl proteins induced by stimulation are indicated by arrows at the left, and positions of size markers (in kilodaltons) are shown at the right.

to IL-3. In the QLWL conserved sequence, mutation of Q-280 to L had only a marginal effect on the response. By contrast, mutation of W-282 to R completely inactivated the receptor for either [^{3}H]thymidine incorporation or long-term growth (data not shown). Mutation of either the conserved S-293 residue to R or E-300 to V had only a marginal effect on the response. Within the conserved LEVL sequence, mutation of either L-306 to R or E-307 to K significantly affected the [^{3}H]thymidine response. In addition, these clones grew only slowly in the presence of Epo but, unlike the W282R mutation, could be maintained for long periods of time (data not shown). In contrast, mutation of E-307 to A had very little effect on receptor function, indicating that it was the introduction of a positively charged residue into the domain that affects function rather than the loss of a negative charge.

Effects of point mutations on induction of tyrosine phosphorylation. Our previous studies demonstrated a correlation between mitogenesis and the induction of tyrosine phosphorylation with a series of carboxyl and internal deletion mutations (24). To extend this observation, we examined the EpoRs with point mutations for induction of tyrosine phosphorylation in response to Epo, using an antiphosphotyrosine monoclonal antibody (4G10) in Western blot analysis (Fig. 4). In our previous studies (24), using the 1G2 monoclonal antibody in immunoprecipitation assays and two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) analysis, Epo stimulation of the wt EpoR consistently induced tyrosine phosphorylation of 92- and 56-kDa proteins. Tyrosine phosphorylation of a 70-kDa protein was more variably seen. Lastly, an inducible, acidic 72-kDa protein was shown to be EpoR and was phosphorylated in response to Epo but not in response to IL-3 (24). Consistent with these studies, inducible phosphorylated proteins of 92 and 56 kDa, as well as proteins in the 70/72-kDa range, were seen by Western blot analysis. The phosphorylation of EpoR was not readily detectable by one-dimensional SDS-PAGE and therefore was evaluated as described below. The Q280L and E307A mutants, which had mitogenic responses of approximately 70 and 100% of that seen with IL-3, showed patterns of tyrosine phosphorylations that were comparable to that seen with IL-3. However, induction of the 70-kDa protein was less apparent in the E307A mutant in response to both Epo and IL-3. Variation in the extent of phosphorylation of the proteins in the 70/72-kDa range is often seen for unknown reasons. With the mutants S293R and E300V, which had mitogenic responses of approximately 90 and 80% of that seen with IL-3, 92-, 72/70-, and 56-kDa proteins were induced but to a lower extent than seen with IL-3. The basis for the lower Epo response in these clones is not known but is probably not related to the levels of receptor expression since S293R expresses 820 sites per cell while E300V expresses 70 sites per cell, compared with the other mitogenically active mutants, which express 240 and 430 sites per cell. In contrast to the mutants mentioned above, no induction of the 92-, 72/70-, or 56-kDa protein was detected in these or additional experiments with the mitogenically impaired mutants W282R, L306R, and E307K.

One of the substrates of tyrosine phosphorylation is the carboxyl region of the EpoR (24, 36). We therefore examined

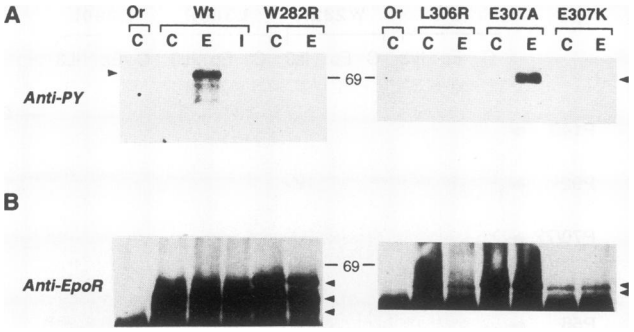

FIG. 5. Induction of tyrosine phosphorylation of the EpoR. Parental DA-3 cells (Or) and those expressing the wt or mutant EpoR, as indicated above the lanes, were deprived of IL-3 for 12 h and stimulated with a saturating concentration of Epo (E) or IL-3 (I) for 10 min or left unstimulated as a control (C). The EpoRs were then immunoprecipitated and resolved on an SDS–6% (left) or SDS–7.5% (right) polyacrylamide gel for Western blot analysis with the antiphosphotyrosine (Anti-PY) antibody 4G10 (A) and subsequently with EpoR antiserum (B) as described in Materials and Methods. Positions of the major species of the tyrosine-phosphorylated receptors (A) and the major EpoR forms (B) are shown by arrows. Positions of 69-kDa size marker are also shown. The bands at the bottom of the gels in panel B represent the rabbit immunoglobulin.

the phosphorylation of the EpoRs with point mutations. In these experiments, EpoR was immunoprecipitated from cells expressing the wt EpoR or one of the mutant receptors following Epo stimulation, and the immunoprecipitates were resolved by SDS-PAGE. The proteins were transferred to nitrocellulose filters and were first probed with the 4G10 antiphosphotyrosine monoclonal antibody. The antibody was then removed by denaturation, and the filters were probed again with the anti-EpoR antiserum to confirm that comparable amounts of the receptors were present on the filters irrespective of Epo stimulation. As shown in Fig. 5, tyrosine phosphorylation of the wt EpoR was specifically induced by stimulation with Epo but not with IL-3; the major species of the tyrosine-phosphorylated receptor appeared as 72 kDa, in accordance with our previous observation (24). Epo stimulation also induced tyrosine phosphorylation of the mutant receptors that are intact in transducing a mitogenic signal (E370A in Fig. 5 and data not shown). In contrast, the mitogenically impaired mutants did not show any detectable phosphorylation (W282R and L306R in Fig. 5) or showed only a faintly detectable phosphorylation in a film exposed longer (E307K; data not shown). Taken together, the results provide further evidence for a correlation of tyrosine phosphorylation and mitogenic activity and identify critical residues within the conserved domain that are required for induction of tyrosine phosphorylation.

Effects of point mutations on expression of immediate-early and early genes following Epo stimulation. One of the consequences of IL-3 stimulation is the induction of the transcription of the immediate-early genes c-*myc* (1, 5, 9), *egr*-1 (unpublished data), and c-*fos* (5) and of the early response genes encoding ODC (1) and TCRγ (34). To determine whether comparable regions of the EpoR were required for mitogenesis, tyrosine phosphorylation, and the induction of immediate-early and early genes, we assessed a series of EpoR mutants for the ability to induce these genes in either DA-3 or 32Dcl cells. For these studies, we utilized the W282R and L306R mutants as well as carboxyl-truncated mutants which lacked 108 (EpoR-H) or 146 (EpoR-S) amino acids. Lastly, we included an internally deleted mutant (EpoR-PB) that lacks amino acids L-281 to E-300 and which is mitogenically inactive. The latter mutants were included to explore the possibility that the EpoR carboxyl domain might uniquely contribute to the signal transduction pathways that affect expression of one or more of the genes examined. Examples of the results are shown in Fig. 6, and a summary of all of the results is presented in Table 2. In either DA-3 or 32Dcl cells containing the wt EpoR, stimulation with Epo induced the transient expression of c-*fos* and induced the expression of c-*myc*, TCRγ, and ODC comparable to the response seen with IL-3. Similarly, in 32Dcl cells with the wt EpoR, Epo induced the expression of the *egr*-1 gene comparably to IL-3. This gene is not inducible in the parental cells or derivatives of the DA-3 cell line. Comparable responses were seen in all cases with clones of cells expressing the mitogenic versus nonmitogenic EpoRs. For example, a carboxyl truncation of 108 amino acids (EpoR-H) which did not affect mitogenicity (24) gave a pattern comparable to that of the wt receptor. In contrast, a carboxyl truncation of 146 amino acids (EpoR-S), which inactivates the receptor when expressed in DA-3 cells, failed to induce c-*fos*, ODC, or TCRγ and caused only a marginal induction of c-*myc*. For unknown reasons, the EpoR-S mutant is mitogenically active in clones of 32Dcl cells, and in these cells, Epo induced c-*myc*, TCRγ, and ODC comparable to the response to IL-3. However, there was no induction of *egr*-1 and only a marginal induction of c-*fos*. The EpoR-PB mutant was not mitogenically active in either cell line, and Epo stimulation of cells containing this mutant did not induce expression of any of the genes examined. Importantly, point mutations that inactivated the receptor for mitogenic activity and for induction of tyrosine phosphorylation (W282R and L306R) also inactivated the receptor for induction of the immediate-early or early gene expression. These results demonstrate that a comparable domain of the receptor is required for mitogenesis, tyrosine phosphorylation, and immediate-early and early gene expression.

DISCUSSION

These studies were initiated to identify critical amino acids in the region of the EpoR that are required for mitogenesis and initially focused on the most conserved amino acids within this region. The mutations within the LEVL sequence, in what has been termed the conserved box 2 region (27), were of particular interest. Two mutations, L306R and E307K, significantly affected receptor function, while the mutation E307A had little effect. Because of the amino acid substitutions examined, it is likely that inactivation of the receptor was due to the introduction of a positively charged residue. Mutation of the equivalent residue of L-306 to P in the IL-2βR inactivated the receptor, suggesting that this region may also be important in the IL-2βR (26). In the IL-6R-associated gp130, carboxyl truncations distal to box 2 were found to not affect function, while carboxyl truncations within or immediately proximal to box 2 inactivate the receptor (27), indicating that box 2 is the most distal region that is required for receptor function. Therefore, the data are consistent with a possible functional role for the box 2 region in the three receptors examined to date.

The most striking mutation obtained in our studies was mutation of the highly conserved W-282 to R, which completely inactivated the receptor. Previous studies with the IL-2βR (26) and the IL-6R-associated gp130 (27) did not

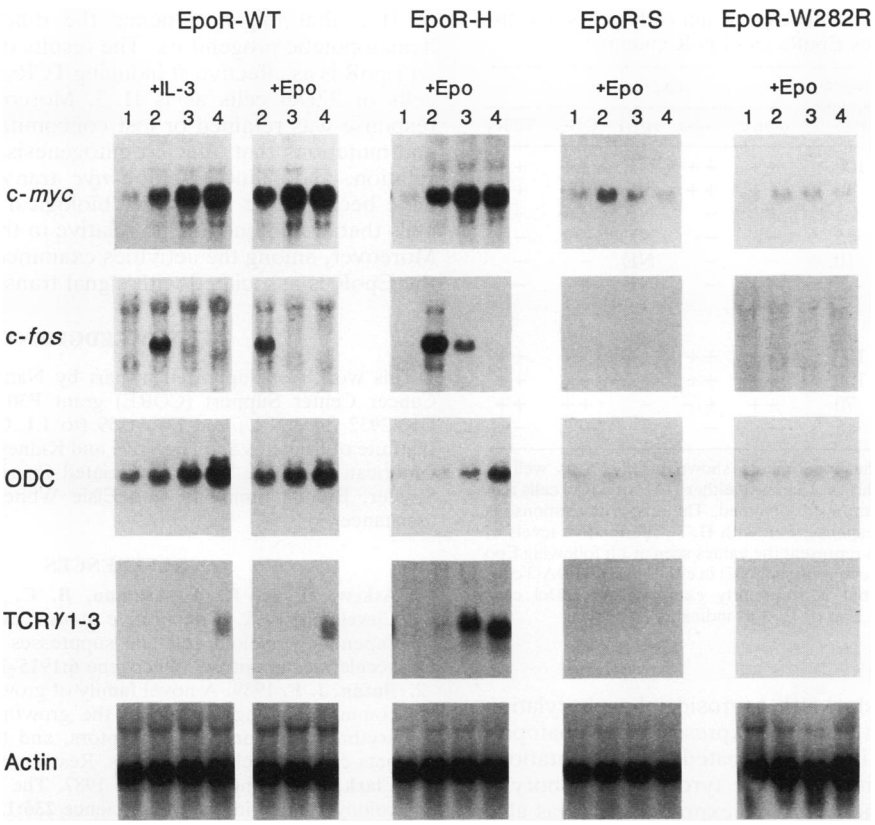

FIG. 6. Induction of immediate-early and early genes by Epo stimulation in DA-3 cells expressing the wt or mutant EpoR. DA-3 cells expressing the wt or mutant EpoR, as indicated above the lanes, were removed from IL-3 for 24 h (lane 1) and stimulated with IL-3 or Epo for 30 min and 2 h (lanes 2 to 4, respectively). Total RNA was extracted from cells and subjected in quadruplication to Northern blot analysis using the indicated probes as described in Materials and Methods.

include mutations of this residue, although the studies with the IL-2βR included a mutation of the carboxyl-flanking L to a P, which did not affect receptor function. Because of the striking similarity of the EpoR and IL-2βR in this region, we introduced the comparable mutation into the IL-2βR (data not shown). This construct was not evaluated in the DA-3 cells because transfection of wt IL-2βR confers only a weak IL-2 responsiveness. However, transfection of wt IL-2βR into 32Dcl cells does confer the ability to respond to IL-2. In 32Dcl cells, a mutated IL-2βR, containing the W-to-R mutation, was functionally intact. Therefore, the conservation of particular amino acids among various hematopoietic growth factor receptors may not necessarily imply a functional role in all receptors. Alternatively, specific amino acid substitutions may not have comparable effects within the context of specific receptors.

Mutations within the conserved S-293–P-294 (S293R) or P-298–E-300 (E300V) amino acids did not affect function. The mutations that were introduced were chosen to optimally change the region by introducing a charged residue or removing a charged residue. In the IL-2βR, changing the position comparable to P-298 to A had only a partial effect on function (26).

Hematopoietic receptors mediate a number of biological functions, and therefore different regions of the receptor may function to couple ligand binding to initiation of specific biological responses. In the case of the EpoR, studies have demonstrated that the carboxyl-terminal domain negatively affects receptor function in Ba/F3 cells (8). Similarly, the IL-2βR contains a carboxyl-terminal domain that binds *lck* but is not required for mitogenesis (13). This concept is also illustrated with receptors for epidermal growth factor, platelet-derived growth factor, or colony-stimulating factor 1, which have been shown to have multiple domains with which signal-transducing proteins interact (19, 33). For example, point mutations in the colony-stimulating factor 1 receptor have been shown to compromise c-*myc* induction and mitogenesis without affecting other responses of ligand binding (31, 32). Our studies examined the effects of point mutations and deletions on the ability of the receptor to induce tyrosine phosphorylation or to induce the expression of immediate-early and early response genes. If different regions were involved in these responses, some of the mutations might be expected to affect one but not another response.

Mutations that eliminated the mitogenic response also eliminated induction of tyrosine phosphorylation. These findings extend our previous studies (24) and further suggest a critical role for tyrosine phosphorylation in receptor function. The observation that point mutations can concomitantly eliminate the phosphorylation of all of the normal substrates, including the phosphorylation of the receptor, suggests that a single domain may be required for association with a kinase or a signal-transducing protein rather than affecting the ability of different substrates to associate with the receptor at multiple sites.

Mutations that eliminated the mitogenic response also eliminated the induction of immediate-early and early gene

TABLE 2. Induction of immediate-early and early genes in cells expressing various EpoRs and EpoR mutants[a]

Cell line	Receptor	Mitogenic response (%)	Expression				
			c-*myc*	c-*fos*	*egr*-1	ODC	TCRγ
DA-3	wt EpoR	100	++	++	NE	++	++
	EpoR-H	40	++	++	NE	++	++
	EpoR-S	<5	+	−	NE	−	−
	EpoR-PB	<5	−	−	NE	−	−
	EpoR-L306R	10	−	−	NE	−	−
	EpoR-W282R	<5	−	−	NE	−	−
32Dcl	wt EpoR	100	++	++	++	++	++
	EpoR-H	100	++	++	++	++	++
	EpoR-S	70	++	+/−	−	++	++
	EpoR-PB	<5	−	−	−	−	−

[a] Results are those from the experiments shown in Fig. 6 as well as additional studies that are not shown. Clones of either DA-3 or 32Dcl cells that expressed the indicated receptors were obtained. The mitogenic response is given as a percentage of the response seen with IL-3. The relative levels of induction of the indicated genes represent the values seen at 1 h following Epo stimulation. The *egr*-1 gene is not expressed (NE) in either parental DA-3 cells or the indicated derivatives but is transiently expressed in 32Dcl cells following induction with either Epo or IL-3 as indicated.

expression. It has been shown that tyrosine phosphorylation is essential for induction of c-*myc* expression in hematopoietic cells (4). Therefore, it was anticipated that the mutations that eliminated the ability to induce tyrosine phosphorylation would also fail to induce c-*myc* expression. It was also anticipated that these mutants would not induce ODC, since the expression of the ODC gene has been linked to c-*myc* expression in hematopoietic cells (1, 9). In both DA-3 and 32Dcl cells, there was an excellent correlation between the loss of a mitogenic response and ability to induce tyrosine phosphorylation in previous studies (24; unpublished studies as well as the studies presented here) and the loss of the ability to induce c-*myc* expression for the deletions and point mutants that were examined.

The responses seen with c-*fos* were less predictable because c-*fos* does not appear to be essential for growth of hematopoietic cells and expression of c-*fos* has not been linked to tyrosine phosphorylation (4). In DA-3 cells, IL-3 induced the transient expression of c-*fos*, while cells expressing the wt EpoR and stimulated with Epo show a temporally similar response. The H-mutant carboxyl truncation of the receptor, which is mitogenically active, resulted in a temporally similar response. In contrast, mitogenically inactive mutants failed to induce c-*fos* expression. Our results with EpoR differ from recent studies with IL-2βR which demonstrated that a carboxyl acidic region as well as a serine-rich region were required for c-*fos* activation (12). Lastly, in general the ability to induce *egr*-1 expression in 32Dcl cells correlated with mitogenic activity, although the absence of expression of this gene in DA-3 cells suggests that it is not required for, or necessarily correlated with, mitogenesis.

IL-3 has been shown to induce the transcription of the nonrearranged TCRγ in a variety of early myeloid lineage, growth factor-dependent cells (34). Transcription was not induced by G-CSF, although G-CSF was mitogenic for the cells. Unlike expression of ODC, expression of the TCRγ locus is not linked to that of c-*myc* (unpublished data). Because of these studies, it had been speculated that induction of TCRγ expression was a biologically unique response to IL-3 that might influence the differentiation of early hematopoietic progenitors. The results demonstrate that the wt EpoR is as effective at inducing TCRγ expression in DA-3 cells or 32Dcl cells as is IL-3. Moreover, this biological response was retained or lost concomitantly with deletions and mutations that affected mitogenesis, tyrosine phosphorylation, and induction of c-*myc* transcription. Thus, we have been unable to identify biological responses in these cells that are unique to Epo relative to the response to IL-3. Moreover, among the activities examined, a single region of the EpoR is associated with signal transduction.

ACKNOWLEDGMENT

This work was supported in part by National Cancer Institute Cancer Center Support (CORE) grant P30 CA21765, by grants DK42932 (to J.N.I.) and DK44159 (to J.L.C.) from the National Institute of Diabetes and Digestive and Kidney Diseases, and by the American Lebanese Syrian Associated Charities. We thank Linda Snyder, Brenda Simmons, and Elsie White for expert technical assistance.

REFERENCES

1. **Askew, D. S., R. A. Ashmun, B. C. Simmons, and J. L. Cleveland.** 1991. Constitutive c-*myc* expression in an IL-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene **6:**1915–1922.
2. **Bazan, J. F.** 1989. A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. Biochem. Biophys. Res. Commun. **164:**788–795.
3. **Clark, S. C., and R. Kamen.** 1987. The human hematopoietic colony-stimulating factors. Science **236:**1229–1237.
4. **Cleveland, J. L., M. Dean, N. Rosenberg, J. Y. Wang, and U. R. Rapp.** 1989. Tyrosine kinase oncogenes abrogate interleukin-3 dependence of murine myeloid cells through signalling pathways involving c-*myc*: conditional regulation of c-*myc* transcription by temperature-sensitive v-*abl*. Mol. Cell. Biol. **9:**5685–5695.
5. **Conscience, J. F., B. Verrier, and G. Martin.** 1986. Interleukin-3-dependent expression of the c-myc and c-fos proto-oncogenes in hemopoietic cell lines. EMBO J. **5:**317–323.
6. **Cosman, D., S. D. Lyman, R. L. Idzerda, M. P. Beckmann, L. S. Park, R. G. Goodwin, and C. J. March.** 1990. A new cytokine receptor superfamily. Trends Biochem. Sci. **15:**265–270.
7. **D'Andrea, A. D., H. F. Lodish, and G. G. Wong.** 1989. Expression cloning of the murine erythropoietin receptor. Cell **57:**277–285.
8. **D'Andrea, A. D., A. Yoshimura, H. Youssoufian, L. I. Zon, J. W. Koo, and H. F. Lodish.** 1991. The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains. Mol. Cell. Biol. **11:**1980–1987.
9. **Dean, J., J. L. Cleveland, U. R. Rapp, and J. N. Ihle.** 1987. Role of myc in the abrogation of IL3 dependence of myeloid FDC-P1 cells. Oncogene Res. **1:**279–296.
10. **Devos, R., G. Plaetinck, J. Van der Heyden, S. Cornelis, J. Vandekerckhove, W. Fiers, and J. Tavernier.** 1991. Molecular basis of a high affinity murine interleukin-5 receptor. EMBO J. **10:**2133–2137.
11. **Fukunaga, R., E. Ishizaka-Ikeda, C. X. Pan, Y. Seto, and S. Nagata.** 1991. Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. **10:**2855–2865.
12. **Hatakeyama, M., A. Kawahara, H. Mori, and H. Shibuya.** 1992. c-*fos* gene induction by interleukin 2: identification of the critical cytoplasmic regions within the interleukin 2 receptor β chain. Proc. Natl. Acad. Sci. USA **89:**2022–2026.
13. **Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S. D. Levin, R. M. Perlmutter, and T. Taniguchi.** 1991. Interaction of the IL-2 receptor with the src-family kinase $p56^{lck}$: identification of novel intermolecular association. Science **252:**1523–1528.
14. **Hatakeyama, M., H. Mori, T. Doi, and T. Taniguchi.** 1989. A

restricted cytoplasmic region of IL-2 receptor b chain is essential for growth signal transduction but not for ligand binding and internalization. Cell **59:**837–845.

15. **Higuchi, R.** 1990. Recombinant PCR, p. 177–183. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, San Diego, Calif.
16. **Horak, I. D., R. E. Gress, P. J. Lucas, E. M. Horak, T. A. Waldmann, and J. B. Bolen.** 1991. T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of $p56^{lck}$. Proc. Natl. Acad. Sci. USA **88:**1996–2000.
17. **Ihle, J. N., A. Rein, and R. Mural.** 1984. Immunological and virological mechanisms in retrovirus induced murine leukemogenesis, p. 95–137. *In* G. Klein (ed.), Advances in viral oncology, vol. 4. Raven Press, New York.
18. **Kipreos, E. T., and J. Y. J. Wang.** 1988. Reversible dependence on growth factor interleukin-3 in myeloid cells expressing temperature sensitive v-abl oncogene. Oncogene Res. **2:**277–284.
19. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252:**668–674.
20. **Kono, T., T. Doi, G. Yamada, M. Hatakeyama, S. Minamoto, M. Tsudo, M. Miyasaka, T. Miyata, and T. Taniguchi.** 1990. Murine interleukin 2 receptor beta chain: dysregulated gene expression in lymphoma line EL-4 caused by a promoter insertion. Proc. Natl. Acad. Sci. USA **87:**1806–1810.
21. **Mathey-Prevot, B., G. Nabel, R. Palacios, and D. Baltimore.** 1986. Abelson virus abrogation of interleukin-3 dependence in a lymphoid cell line. Mol. Cell. Biol. **6:**4133–4135.
22. **Metcalf, D.** 1989. The molecular control of cell division, differentiation commitment and maturation in hemopoietic cells. Nature (London) **339:**27–30.
23. **Migliaccio, G., A. R. Migliaccio, B. L. Kreider, G. Rovera, and J. W. Adamson.** 1989. Selection of lineage-restricted cell lines immortalized at different stages of hematopoietic differentiation from the murine cell line 32D. J. Cell Biol. **109:**833–841.
24. **Miura, O., A. D'Andrea, D. Kabat, and J. N. Ihle.** 1991. Induction of tyrosine phosphorylation by the erythropoietin receptor correlates with mitogenesis. Mol. Cell. Biol. **11:**4895–4902.
25. **Miyazaki, T., M. Maruyama, G. Yamada, M. Hatakeyama, and T. Taniguchi.** 1991. The integrity of the conserved 'WS motif' common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction. EMBO J. **10:**3191–3197.
26. **Mori, H., E. L. Barsoumian, M. Hatakeyama, and T. Taniguchi.** 1991. Signal transduction by interleukin 2 receptor beta chain: importance of the structural integrity as revealed by site-directed mutagenesis and generation of chimeric receptors. Int. Immunol. **3:**149–156.
27. **Murakami, M., M. Narazaki, M. Hibi, H. Yawata, K. Yasukawa, M. Hamaguchi, T. Taga, and T. Kishimoto.** 1991. Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc. Natl. Acad. Sci. USA **88:**11349–11353.
28. **Pierce, J. H., P. P. Di Fiore, S. A. Aaronson, M. Potter, J. Pumphrey, A. Scott, and J. N. Ihle.** 1985. Neoplastic transformation of mast cells by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. Cell **41:**685–693.
29. **Quelle, D. E., and D. M. Wojchowski.** 1991. Localized cytosolic domains of the erythropoietin receptor regulate growth signaling and down-modulate responsiveness to granulocyte-macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. USA **88:**4801–4805.
30. **Quelle, F. W., and D. M. Wojchowski.** 1991. Proliferative action of erythropoietin is associated with rapid protein tyrosine phosphorylation in responsive B6SUt.EP cells. J. Biol. Chem. **266:**609–614.
31. **Roussel, M. F., J. L. Cleveland, S. A. Shurtleff, and C. J. Sherr.** 1991. Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signalling. Nature (London) **353:**361–363.
32. **Roussel, M. F., S. A. Shurtleff, J. R. Downing, and C. J. Sherr.** 1990. A point mutation at tyrosine-809 in the human colony-stimulating factor 1 receptor impairs mitogenesis without abrogating tyrosine kinase activity, association with phosphatidylinositol 3-kinase, or induction of c-fos and junB genes. Proc. Natl. Acad. Sci. USA **87:**6738–6742.
33. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212.
34. **Weinstein, Y., K. Morishita, J. L. Cleveland, and J. N. Ihle.** 1989. Interleukin 3 (IL-3) induces transcription from non-rearranged gamma T cell loci in IL-3-dependent cell lines. J. Exp. Med. **169:**2059–2071.
35. **Yoshimura, A., A. D. D'Andrea, and H. F. Lodish.** 1990. Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. Proc. Natl. Acad. Sci. USA **87:**4139–4143.
36. **Yoshimura, A., and H. F. Lodish.** 1992. In vitro phosphorylation of the erythropoietin receptor and an associated protein, pp130. Mol. Cell. Biol. **12:**706–715.
37. **Zon, L. I., J.-F. Moreau, J.-W. Koo, B. Mathey-Prevot, and A. D. D'Andrea.** 1992. The erythropoietin receptor transmembrane region is necessary for activation by the Friend spleen focus-forming virus gp55 glycoprotein. Mol. Cell. Biol. **12:**2949–2957.