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# Localized cytosolic domains of the erythropoietin receptor regulate growth signaling and down-modulate responsiveness to granulocyte–macrophage colony-stimulating factor

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ABSTRACT **Erythrocyte development in mammals depends in part upon the interaction of the glycopeptide hormone erythropoietin (EPO) with cell surface receptors on committed erythroid progenitor cells. Both this factor and an EPO receptor polypeptide previously have been cloned, yet little is presently understood concerning molecular mechanisms of receptor activation and signal transduction. To identify cytosolic receptor domains necessary for signaling, we have compared the activities of a series of deletionally mutated EPO receptor constructs by their expression in interleukin 3-dependent, myeloid FDC-P1 cells. EPO-induced growth was transduced efficiently in these cells by the full-length receptor (507 amino acids), and no measurable loss in activity resulted from the deletion of up to 111 carboxyl-terminal residues. In contrast, the deletion of 44 additional residues led to a dramatic loss (86.3% ± 7.8%; mean ± SD) in the ability of this receptor to mediate EPO-induced growth, thus indicating that residues between Gly-352 and Met-396 constitute a functionally critical cytosolic subdomain. Interestingly, the expression of full-length EPO receptors in FDC-P1 cells also led to a selective inhibition of normal proliferative responsiveness to the alternative hematopoietic factor granulocyte–macrophage colony-stimulating factor. Moreover, this inhibition was progressively reversed in forms of the EPO receptor in which distal cytosolic residues were sequentially deleted. These results suggest that EPO receptors normally may trans-modulate components in the pathway of granulocyte–macrophage colony-stimulating factor-induced proliferation and that this down-modulation, as exerted by intact EPO receptors, may play a role in promoting erythroid commitment during myeloid blood cell development.**

The hematopoietic growth factor erythropoietin (EPO) promotes the proliferation of erythroid progenitor cells in marrow (1), spleen (2), and fetal liver (3) and is required for their subsequent terminal differentiation to circulating erythrocytes (4). Cell surface receptors for EPO have been identified on erythroid cells (5, 6) and leukemic cell lines (7, 8), and a cDNA encoding an EPO receptor polypeptide has been cloned from Friend murine erythroleukemia cells by expression (9). Recently, this cDNA has been used to isolate the human EPO receptor gene, which is highly homologous in coding sequence and predicted size with the murine receptor (10, 11). Importantly, these cDNAs have been shown to encode functional receptors that mediate EPO-induced growth when expressed in heterologous lymphoid Ba/F3 cells (10, 12).

Despite this progress, molecular mechanisms involved in EPO receptor activation and signal transduction presently are poorly understood. Recently, this receptor has been classified with the receptors for interleukin (IL)-2 (13), IL-3 (14), IL-6 (15), granulocyte–macrophage colony-stimulating factor (GM-CSF) (16), and IL-4 (17, 18) into an emerging superfamily of cytokine receptors (19, 20). This classification is based largely on recognizable homologies within extracellular domains, with no identified catalytic activities encoded within the cytoplasmic domains of these receptors. However, possible similarity in mechanisms of growth signal transduction has been suggested to exist, at least among the EPO, IL-2, IL-3, and GM-CSF receptor systems based on similar ligand-activated protein-tyrosine phosphorylation events (21–24). EPO, for example, induces the tyrosine phosphorylation of plasma membrane-associated peptides in B6SUt.EP cells; these peptides are essentially identical in molecular weight to those phosphorylated in response to IL-3 (21).

Certain of these receptors have also been proposed to occur complexed with accessory polypeptides. For instance, the IL-6 receptor apparently may transduce growth signaling through direct interaction with an associated transmembrane polypeptide, gp130 (25). Similarly, a second subunit of the GM-CSF receptor recently has been identified (26); it forms a high-affinity receptor complex when coexpressed with a previously cloned receptor subunit (16). The possible existence of accessory polypeptides in the EPO receptor system has likewise been proposed based on binding and cross-linking studies (9, 27, 28). Also, it recently has been demonstrated that the gp55 glycoprotein of the Friend virus directly binds to and activates the murine EPO receptor, thus possibly mimicking the actions of a putative associated protein (12, 28). However, neither the identity of these cross-linked polypeptides nor their possible site(s) of association with the EPO receptor is known.

Toward identifying functional domains of the murine EPO receptor that are involved in growth signal transduction, we have deletionally mutated cytoplasmic regions and have assayed the function of these mutated receptors by expression in heterologous murine myeloid FDC-P1 cells (29). Through this approach, a minimal membrane-proximal region is delineated that is essential for the efficient transduction of EPO-induced proliferation. In contrast, the terminal residues of the cytoplasmic domain, while dispensible for EPO-induced growth, are shown to mediate a selective down-modulation of the normal responsiveness of FDC-P1 cells to GM-CSF. The significance of this trans-modulation effect is discussed, as are implications for possible overlap in the immediate pathways of EPO- and GM-CSF-induced cell growth.

## MATERIALS AND METHODS

**Cell Culture, Growth Factors, and Proliferation Assays.** Murine FDC-P1 cells (myeloid cells that are normally completely dependent on IL-3 or GM-CSF for growth) (29) were maintained in Dulbecco's modified minimum essential me-

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Abbreviations: EPO, erythropoietin; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor.

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dium supplemented with 10% fetal bovine serum, 0.01 mM 2-mercaptoethanol, and 2.5% conditioned medium from WEHI-3B cells (30). Proliferation assays were performed in 96-well plates ($3 \times 10^4$ cells per 0.1 ml; 18–20 hr) using purified growth factors [recombinant human EPO, 150,000 units/mg, Johnson Pharmaceutical Research Laboratories (La Jolla, CA); recombinant murine GM-CSF, Immunex (Seattle); synthetic murine IL-3, Biomedical Research Center (Vancouver)]. Cells were labeled for 2 hr with [$^3$H]thymidine (10 $\mu$Ci/ml; 1 Ci = 37 GBq).

**EPO Receptor cDNA Constructs.** Plasmids used in these studies were prepared from the murine EPO receptor cDNA clone 190 in pXM (9). To directionally delete 3′ coding regions of this cDNA, a 0.1-kilobase (kb) *Cla* I–*Xho* I fragment from the 3′ noncoding region was excised, and an adaptor containing *Nru* I and *Not* I sites, plus translational stop codons in all reading frames, was inserted. Progressive deletion toward the 5′ terminus using exonuclease III (Erase-A-Base System; Promega) was initiated at the *Nru* I site after blunt ending the *Not* I site with thiol-dNTPs. Deletion constructs then were blunt ended by using S1 nuclease and were self-ligated. Sizes of the mutated cDNAs were determined by nucleotide sequencing. The deletion mutant pXM-stop was constructed by excising the 0.6-kb *Apa* I–*Cla* I fragment from pXM190 and inserting a 14-base-pair *Xba* I stop linker (Pharmacia) at these blunt-ended sites. In the amino-terminal deletion mutant pXM190N, the EPO receptor cDNA was cloned into pUC19, and a 0.365-kb *Sac* II–*Bss*HII fragment was deleted. In order to restore the correct reading frame, a synthetic adaptor [5′-d(pGGGTCTAGAGCGA)-3′] was inserted, and this altered cDNA then was cloned into pXM. The EPO receptor construct pXM141 (9) contains a translational stop mutation at Ile-479 and was used without further modification.

**Gene Transfer.** EPO receptor cDNA constructs were cotransfected with pSV2-neo (31) at a 5:1 molar ratio into FDC-P1 cells ($10^7$ cells/ml in phosphate-buffered saline) by high-voltage electroporation (75 $\mu$g of plasmid DNA per ml, 500 V/cm, and 950 $\mu$F; geneZAPPER, IBI). FDC-P1 cells were also cotransfected with pSV2-neo and pXM as a control. Three to five independent electroporations were performed for each construct. Transfected cells were selected by using G418 at 1 mg/ml (Geneticin; GIBCO). Subsequently, selection of EPO-dependent cells was accomplished in medium lacking conditioned medium from WEHI-3 cells and containing EPO at 5 units/ml. Sublines were established by dilutional cloning.

**Northern Analyses.** Total RNA was isolated by using a guanidinium isothiocyanate/CsCl gradient sedimentation procedure (32). After electrophoresis in formaldehyde/agarose gels, RNA (10 $\mu$g) was blotted and hybridized to probes in the presence of 50% formamide (33). Probes were labeled using [$\alpha$-$^{32}$P]dCTP, random hexameric primers, and the Klenow fragment of DNA polymerase I [EPO receptor cDNA, 1.5 kb *Xho* I fragment of pXM190; murine actin, 1.0 kb *Pst* I fragment of pBA.1 (34)].

**Western Blot Analysis of Cell Surface Receptors.** In analyses of EPO receptor expression at the cell surface, cells ($4 \times 10^7$) were washed in phosphate-buffered saline, and plasma membrane fractions were isolated (21). Western blot analyses were performed as described by Towbin *et al.* (35) by using antisera prepared in rabbits against synthetic peptides with sequences corresponding to the predicted amino-terminal (Ala-24 to Lys-37) and carboxyl-terminal (Ser-490 to Cys-506) domains of the murine EPO receptor (36).

## RESULTS

**Delineation of a Minimal Functional Cytosolic Domain of the EPO Receptor.** In primary experiments, FDC-P1 cells transfected with a full-length EPO receptor cDNA (i.e., FDC-ER cells) were observed to proliferate in response to EPO and to acquire growth dependence for this factor. EPO dependence was established rapidly (within 1 or 2 days) and was observed in all clones examined. No induction of erythroid differentiation was observed in FDC-ER cells with no detectable expression of either early (GATA-1) or late (globins) erythroid genes, and normal responsiveness to IL-3 (the original factor of dependency) was fully retained. In contrast, a substantial down-modulation of normal responsiveness to GM-CSF was observed (see below).

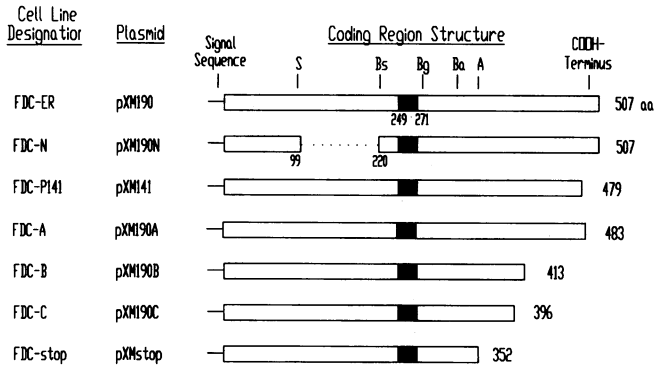


FIG. 1. EPO receptor constructs and derived cell lines. The peptide structures of deletionally mutated EPO receptors as predicted from cDNA constructs within the designated pXM expression plasmids are diagrammed. Positions of the predicted transmembrane domain (filled box), lengths of the truncated receptor peptides, and cDNA restriction sites are indicated. S, *Sac* II; Bs, *Bss*HII; Bg, *Bgl* II; Ba, *Bam*HI; A, *Apa* I. Designations of the cell lines expressing these various receptor forms are also indicated. aa, amino acids.

To identify cytosolic regions of the EPO receptor that are involved in growth signaling, a series of deletional mutants was constructed (Fig. 1), expressed in FDC-P1 cells, and then assayed for efficiency in mediating EPO-induced growth

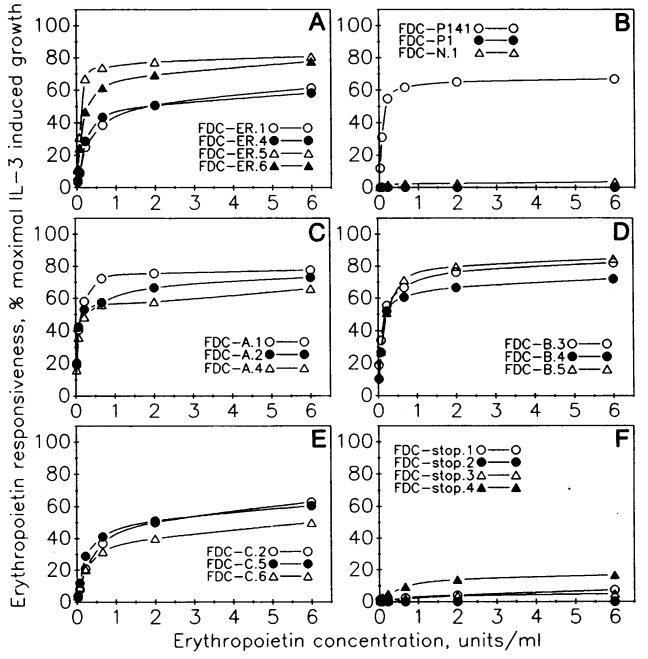


FIG. 2. EPO responsiveness of FDC-P1 cells expressing truncated EPO receptor forms. Rates of EPO-induced [$^3$H]thymidine incorporation were assayed and were normalized by direct comparison to maximal growth rates in response to IL-3 (4 nM). Cell lines expressing mutated receptors are as designated in Fig. 1. Subclones are indicated by decimal points and numbers. EPO dose–response curves for various cell lines and subclones are shown.

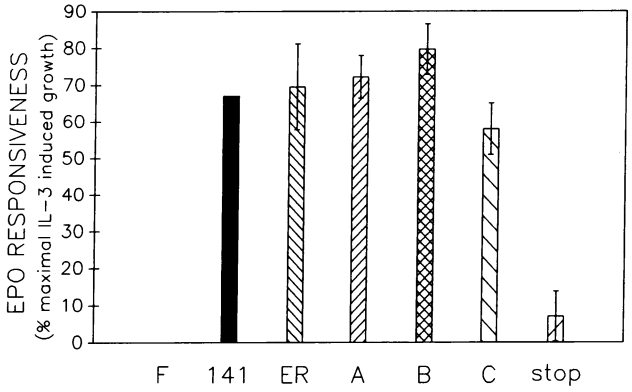


FIG. 3. Comparison of maximal EPO responsiveness among cell lines based on the average responsiveness of subclones from the data in Fig. 2. The abbreviations for cell lines are F, FDC-P1; 141, FDC-P141; ER, FDC-ER; A, FDC-A; B, FDC-B; C, FDC-C; stop, FDC-stop. FDC-P1 cells are bone marrow-derived, nontumorigenic myeloid cells that normally show complete dependence on IL-3 or GM-CSF for growth (29).

(Fig. 2). Growth signaling was not affected significantly by the deletion of as many as 111 amino acids from the terminal cytoplasmic domain (Fig. 2 *B–E*; FDC-P141, FDC-A, FDC-B, and FDC-C cells), as compared to the activity of full-length receptor (FDC-ER cells; Fig. 2*A*). In contrast, the deletion of 155 carboxyl-terminal residues (i.e., pXMstop construct; FDC-stop cells; Fig. 2*F*) dramatically decreased the efficiency of EPO-induced proliferation to only 13.7% ± 7.8% (mean ± SD) of the maximal response in cells expressing longer receptor forms (Fig. 3). Thus, a cytoplasmic subdomain of the EPO receptor contained within the region Gly-352 to Met-396 is indicated to be critically involved in growth signal transduction. Since responsiveness to IL-3 was essentially equivalent for all sublines, responsiveness to EPO in these analyses is expressed as percent of the maximal growth rate induced by IL-3. This normalization allows for comparison of EPO responsiveness among clonal lines expressing each form of receptor and among sets of clones expressing different forms.

In part, varied levels of receptor expression might be anticipated to contribute to differential EPO responsiveness among transfected FDC-P1 cell lines. However, this apparently is not a significant factor: Northern analyses of EPO receptor transcripts among clonal lines expressing a given receptor form confirm a substantial range in expression levels (Fig. 4), yet maximal EPO responsiveness among these clones is comparatively uniform (Fig. 2). Thus, differences in forms of receptor and not variable levels of expression are considered to account for the observed differences in receptor activity.

Other possible explanations for the limited activity of extensively truncated EPO receptors in FDC-stop cells might

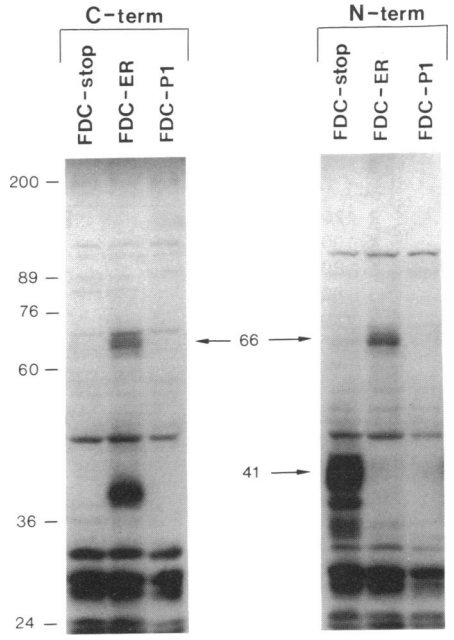


FIG. 5. Western blot analyses of EPO receptors in plasma membrane fractions of FDC-ER and FDC-stop cells. Plasma membranes isolated from FDC-ER, FDC-stop, and FDC-P1 cells (negative control) were subjected to SDS/PAGE (9.5% gel) and were assayed for EPO receptors by Western blotting with antibodies to carboxyl-terminal (*Left*) or amino-terminal (*Right*) peptides. Cell lines, molecular size markers ($M_r \times 10^{-3}$), and the positions of full-length ($M_r$, 66,000) and truncated ($M_r$ 41,000) EPO receptors are indicated.

involve instability or inefficient transport to the cell surface. For example, point mutations of the insulin receptor recently have been shown to impair receptor transport (37, 38). However, Western blotting of EPO receptors in plasma membrane fractions (Fig. 5) indicated levels of cell surface receptor expression, in fact, to be significantly higher in FDC-stop cells ($M_r$ 41,000; Fig. 5 *Right*) as compared to levels in FDC-ER cells ($M_r$ 65,000 and $M_r$ 67,000; Fig. 5 *Left* and *Right*). Thus, inefficient expression apparently does not explain the lower activity of this mutated EPO receptor form. In FDC-ER cells the $M_r$ 39,000 receptor polypeptide was reproducibly detected by antibodies against the carboxyl-terminal domain and is thought to result from proteolysis at a specific site within the extracellular domain.

**EPO Receptor-Mediated Down-Modulation of GM-CSF Responsiveness.** Preliminary experiments using nonclonal lines indicated that the expression of full-length EPO receptors in FDC-P1 cells resulted in a substantial loss of normal proliferative responsiveness to GM-CSF (F. W. Quelle and D.M.W., unpublished results). Analyses of clonal lines sub-

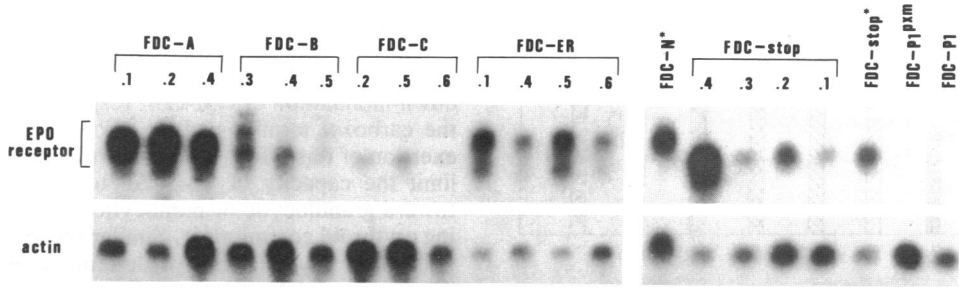


FIG. 4. EPO receptor transcript levels in cells expressing mutated receptor forms. Levels of EPO receptor transcripts were assayed by Northern blotting using actin as an internal standard. Designations of cell lines and subclones are as indicated in Fig. 1 and the legend to Fig. 2. Asterisks indicate nonclonal lines. FDC-P1$^{pxm}$, FDC-P1 cells transfected with pXM vector.

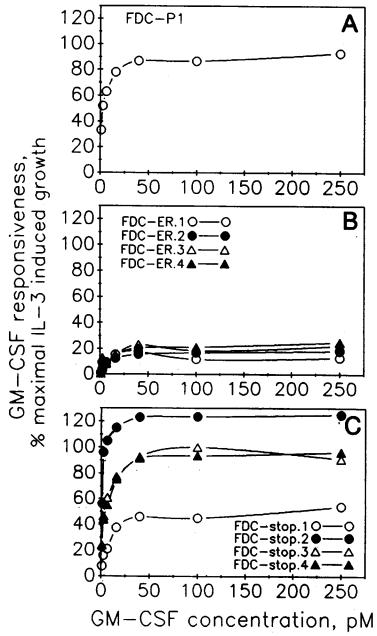


FIG. 6. GM-CSF responsiveness in FDC-P1 cells expressing mutated EPO receptor forms. Rates of GM-CSF-induced [$^3$H]thymidine incorporation were assayed and were normalized by direct comparison to maximal growth rates induced by IL-3 (4 nM). Cell lines and subclones are as designated in Fig. 1 and the legend to Fig. 2. GM-CSF dose–response curves for FDC-P1 cells and subclones of FDC-ER and FDC-stop cells are shown.

stantiated this finding (Fig. 6 *A* and *B*) and indicated a selective loss in GM-CSF responsiveness of ≈90%, with no measurable effect on normal responsiveness to IL-3. Furthermore, analyses of clonal lines expressing truncated forms of the EPO receptor interestingly revealed that inhibition of GM-CSF responsiveness progressively was reversed as larger regions were deleted from the carboxyl terminus (Figs. 6 and 7). In Fig. 7 this effect is illustrated as the mean of the maximal response to GM-CSF relative to the maximal IL-3 growth rates for each EPO receptor construct. Notably, reversal of GM-CSF down-modulation by EPO receptor truncation was observed not only for minimally active receptor forms (construct pXMstop; FDC-stop cells) but also in forms that efficiently transduce EPO-induced growth (i.e., Figs. 2 and 3; constructs pXM141, pXM190A, pXM190B, and pXM190C; FDC-P141, FDC-A, FDC-B, and FDC-C cells). Down-modulation of GM-CSF responsiveness was also observed for EPO receptor forms that were mutated in the

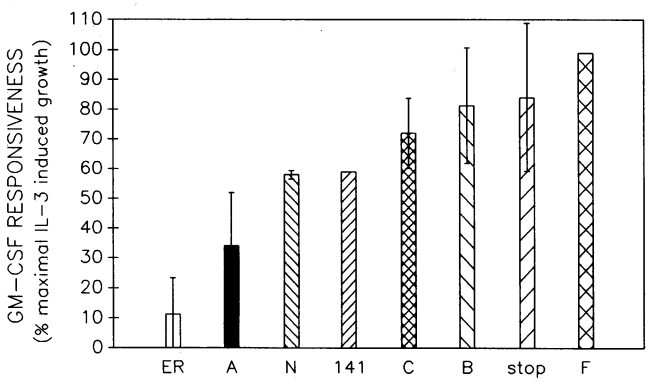


FIG. 7. Comparison of GM-CSF responsiveness among cell lines based on the average response of subclones. Abbreviations for cell lines are as in the legend to Fig. 3, with the exception of subclones FDC-N.1 and FDC-N.2 (N, amino-terminal receptor mutant).

extracellular domain (construct pXM190N; FDC-N cells) and were otherwise nonfunctional (Fig. 2*B*).

## DISCUSSION

The present studies serve to identify two distinct functional domains within the predicted cytosolic region of a recently cloned murine EPO receptor peptide. First, through the expression in FDC-P1 cells of EPO receptor forms that are truncated at the carboxyl terminus, a limited cytosolic domain was delineated (Gly-352 to Met-396) that is required for efficient transduction of EPO-induced cell growth. In contrast, the distal portion of the cytosolic domain (i.e., Met-396 to Ser-507), while dispensable for growth signaling, was shown to exert a selective inhibitory effect on the normal proliferative responsiveness of FDC-P1 cells to GM-CSF, with no measurable effect on normal responsiveness to IL-3.

With regard to the cytosolic subdomain Gly-352 to Met-396, truncation of this region is shown to nearly abrogate EPO-induced growth. Since no recognizable enzymatic activities are encoded by the EPO receptor and since little is understood concerning transduction events, the precise function of this domain presently is undefined. One direct role might possibly involve interaction with immediate activators in the induced pathway to cell growth. Recently, EPO has been shown to rapidly activate protein-tyrosine phosphorylation in responsive B6SUt.EP cells (21). By analogy, the receptors for CD4 and CD8 have been shown to interact directly via cytoplasmic domains with the ligand-activated tyrosine kinase p56$^{lck}$ (39), and tyrosine kinase activity has been found specifically associated with an immunoprecipitated IL-2 receptor complex (40). Also, it is interesting that the subdomain of the EPO receptor including Ser-316 to Gln-341 shares limited homology with a similarly localized domain of the IL-2 receptor $\beta$ chain (41), which has been shown to be essential for IL-2-induced cell growth (42).

Although a role for the delineated cytosolic domain Gly-352 to Met-396 of the EPO receptor is emphasized, receptor forms that are truncated within this region nonetheless retain limited activity. Thus, whether activity is retained in further truncated forms merits investigation. By comparison, secreted forms of the IL-6 receptor have been shown to transduce growth signaling, apparently via interaction with an accessory transmembrane peptide, gp130 (25). In similar studies of the nerve growth factor receptor, deletion of specified cytoplasmic sequences led to a loss of responsiveness and high-affinity binding, a result that has been proposed to implicate the existence of accessory peptides (43). Whether the diminished activity of FDC-stop EPO receptor mutants might likewise involve altered binding properties has not been assessed yet. However, this is a critical question since it has been suggested that high-affinity forms are physiologically active (44–46). Also, it has recently been reported that increased proliferative activity (3- to 5-fold) of the murine EPO receptor results from limited truncation at the carboxyl terminus (i.e., from Thr-441 to Ser-507) (47). However, this effect was not observed in the present study.

Expression studies of mutant EPO receptors also revealed a second interesting feature of EPO receptor function (i.e., down-modulation of GM-CSF responsiveness as exerted by the carboxyl terminus of the EPO receptor). Biologically, exertion of this effect in early normal progenitor cells might limit the capacity of GM-CSF to stimulate myeloid cells toward granulocytic or monocytic pathways, while promoting erythroid commitment. Similar receptor-mediated mechanisms for myeloid lineage determination have previously been proposed based on (*i*) ligand-dependent trans down-modulation of receptors for IL-3, GM-CSF, and G-CSF (48, 49) and (*ii*) GM-CSF or IL-3 posttranscriptional control of M-CSF receptor expression (50). However, trans-modula-

tion of GM-CSF responsiveness by EPO receptors as presently observed in FDC-ER cells differs from these mechanisms in that this effect apparently is ligand independent.

Mechanistically, down-modulation of GM-CSF responsiveness in FDC-ER cells is shown to be mediated by the carboxyl-terminal region of the murine EPO receptor, a domain that apparently is not required for EPO-induced growth. This effect is specific (normal responsiveness to IL-3 is unaffected) and apparently is not attributable to possible commitment toward erythroid differentiation since neither early (i.e., GATA-1) nor late (i.e., globins) erythroid genes are detectably activated in FDC-ER cell lines as maintained in the presence or absence of EPO. Furthermore, this effect does not require functional EPO receptors, since inactive forms that are mutated in the extracellular domain also down-modulate this GM-CSF response.

Two alternative mechanisms for the observed loss of GM-CSF responsiveness are then considered possible. Expression of EPO receptors in FDC-P1 cells might inhibit the expression of receptors for GM-CSF. However, since the murine GM-CSF receptor has not yet been cloned and since a recently isolated human cDNA clone does not hybridize to murine receptor transcripts (16), this is not presently assayable at the transcriptional level. Alternatively, it is possible that EPO receptors may compete with GM-CSF receptors for a limiting component in their immediate pathways of induced cell growth. The nature of this component in the EPO receptor system presently is undefined, yet the existence of accessory polypeptides has been suggested based on binding and cross-linking studies (9, 27, 28). Interestingly, a putative second subunit of the human GM-CSF receptor has recently been cloned and has been shown to be involved in the formation of high-affinity GM-CSF receptors (26). Further analyses of the EPO and GM-CSF receptor systems, including the roles of their cytosolic domains, associated activatable kinases, and accessory polypeptides, should provide important insight toward defining intracellular pathways of induced growth. These studies should also elucidate the nature of the possible direct interaction between these receptors during myeloid hematopoiesis.

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