REVIEW ARTICLE

Structure, Function, and Activation of the Erythropoietin Receptor

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ERYTHROPOIETIN (EPO) is the principal growth factor that promotes the viability, proliferation, and differentiation of mammalian erythroid progenitor cells, functions that are transduced by the specific cell surface EPO receptor (EPO-R). In contrast to many other hematopoietic growth factors, EPO primarily affects relatively mature erythroid cells, although it may also influence the growth and behavior of other cells. The recent cloning of the EPO-R gene has greatly facilitated the molecular analysis of the structure and function of this receptor, and provided a fundamental link to earlier biochemical and cellular studies of erythropoiesis. Here we describe recent studies on the structure, function, and activation of this receptor. One focus will be on mechanisms by which the activated receptor can induce cellular proliferation in the absence of EPO. This can be achieved by binding of a retrovirus envelope glycoprotein to the EPO-R or by a specific point mutation in the exoplasmic domain of the EPO-R, EPO-independent activation of the EPO-R can contribute to the development of erythropoiesis in animal model systems. A second focus will be on the structure and control of expression of the EPO-R gene.

MAMMALIAN ERYTHROPOIESIS
AND ERYTHROID CELL LINES

Within the fetal liver and adult bone marrow (BM), pluripotential hematopoietic stem cells generate erythroid progenitors. The earliest cells identified ex vivo as erythroid progenitors are the slowly proliferating burst-forming unit-erythroid (BFU-E), which are not responsive to EPO and do not express the EPO-R. After 48 to 72 hours of growth in the presence of interleukin-3 (IL-3),1,2 granulocyte-macrophage colony-stimulating factor (GM-CSF),1,2 or Steel factor (SF),3 "mature" BFU-E develop that express the EPO-R and are weakly responsive to EPO.4 After another 4 to 5 days in culture, these cells give rise to smaller colony-forming units-erythroid (CFU-E). CFU-Es are highly responsive to EPO and generate erythroblast colonies in 7 days (in humans) or 2 days (in mice).5,6 The sensitivity of these erythroid precursor cells to EPO is transient; it declines gradually with increasing maturation such that cells beyond the erythroblast stage are no longer dependent on EPO, and concomitantly display fewer EPO-Rs per cell.7

Human and mouse CFU-E cells and mouse fetal liver cells proliferate and differentiate in response to EPO, as do splenic erythroid precursors derived from mice infected with an anemic strain of Friend virus (SFFV-A; see below). In culture such cells express approximately 300 to 1,000 EPO-binding sites on their surfaces: 20% are high affinity (kd = 100 pmol/L), and 80% are considered low affinity (kd = 600 pmol/L).8,9 In contrast, EPO-Rs of only a single affinity (kd = 100 to 200 pmol/L) were found during differentiation of human BFU-Es to reticulocytes.12 Many features of mammalian erythropoiesis are recapitulated by immortalized cell lines, including EPO-independent proerythroblasts isolated from the spleens of mice infected with a polycythemic strain of Friend virus (SFFV-P). These cells, known collectively as murine erythroleukemia (MEL) cells, generally express less than 1,000 surface EPO-binding sites per cell, but, unlike CFU-Es, they express receptors of only a single affinity (240 to 1,000 pmol/L).11 However, they differ from normal progenitors in at least two respects: they do not require EPO for proliferation nor do they differentiate in its presence, although a number of chemicals, such as dimethylsulfoxide and hexamethylene-bis-acetamide, successfully induce erythroid differentiation. In cells expressing both high- and low-affinity receptors, only the high-affinity form might be responsible for the biologic effects of EPO, as few low-affinity receptors are expected to be occupied at physiologic concentrations of EPO.9,11

FUNCTIONAL DOMAINS OF THE EPO-R

The cDNA of the murine EPO-R encodes a protein of 507 amino acids that contains a single hydrophobic membrane spanning domain (Fig 1). The amino acid sequence of the human EPO-R13 is 82%, identical to that of the mouse protein.14 Following the cloning of the cDNA for the EPO-R, cDNAs encoding other hematopoietic growth factor receptors were isolated in rapid succession. Sequence analysis identified a new family of receptors that share two distinctive features in their exoplasmic domains: a set of four conserved Cys residues, and a five-residue motif located close to the transmembrane domain, Trp-Ser-X-Trp-Ser (WSXWS), where X represents any amino acid.15-18 Members of this family include receptors for EPO, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF, leukemia inhibitory factor (LIF), growth hormone (GH), prolactin, neurotrophic trophic factor (CNTF), and the env gene of mouse myeloproliferative leukemia virus, v-mpl, along with its cellular homolog, c-mpl.15-23

Expression of the EPO-R in normally IL-3-dependent cell lines such as Ba/F3 or 32D allows cell proliferation in the presence of either EPO or IL-3, indicating that the cloned EPO-R is capable of generating a proliferative signal in these cells.24,25 In an effort to delineate the domains essential for signal transduction, we and others26-28 have introduced several deletions in the cytoplasmic tail of the EPO-R. Two regions
of opposing function were defined: a membrane-proximal region of about 100 amino acids that is sufficient for transduction of proliferative signals, and a distal region that downmodulates such signals. The proximal region is homologous to the corresponding segment of the IL-2-R β-chain, which is also sufficient for signal transduction.29

Deletion of the carboxy-terminal 40 to 90 amino acids of the EPO-R allows Ba/F3 cells to proliferate in as little as 1 pmol/L EPO, one tenth that required for proliferation of cells expressing the wild-type EPO-R. Because the deletion had no effect on the number or affinity of cell-surface EPO-Rs, occupancy of fewer cell-surface receptors by EPO than in cells expressing the wild-type EPO-R was sufficient to generate the growth-promoting intracellular signal. Thus, the carboxy-terminal region of the cytoplasmic domain is thought to downmodulate the intracellular growth signal (see below).27

Interestingly, this inhibitory region also appears to downmodulate the proliferative action of GM-CSF in myeloid progenitor FDC-P1 cells transfected with the EPO-R cDNA,28 suggesting the possibility of "cross-talk" between this family of receptors. The carboxy-terminal region includes putative phosphorylation sites or binding sites for a Tyr kinase (Fig 1).30 The precise residues involved and the mechanism for downmodulation of the EPO-R and GM-CSF-R remain to be established.

The conserved WSXWS motif was predicted to be an essential component of the ligand-binding site of cytokine receptors.18,19 The motif is also found in complement precursor proteins (C7, C8, and C9), thrombospondin, and properdin, and is thought to mediate protein–protein interactions.19 Lymphoid Ba/F3 cells expressing mutant EPO-Rs lacking part or all of this motif, or containing a single Gly insertion between the two WS residues, express few, if any, EPO-Rs on the cell surface and do not grow in the presence of EPO.31 Biochemical and immunohistochemical analysis show that the mutant receptors are retained in the endoplasmic reticulum and are unable to bind EPO. In the case of the IL-2-R, mutation of either or both Ser residues in the WSXWS motif of the IL-2-R β-chain does not affect binding of IL-2 or its ability to trigger cell proliferation, whereas substitution of either of the two Trp residues to Gly or Ser destroys IL-2 binding.32 Thus, the Trp residues as well as the spacing between them seem to be critical features of receptor structure and function.

The three-dimensional structure of the exoplasmic domain of the related GH receptor consists of two sub-domains, each of which contains seven β-sheets (Fig 2).33 The four conserved Cys residues form two disulfide bonds that stabilize the structure of the membrane-distal sub-domain. The sequence homologous to WSXWS is not present in the ligand-binding region. Rather, it is an integral component of the membrane-proximal subdomain, consistent with the finding that EPO-R molecules mutant in this region are unable to fold into structures that either bind EPO or exit the endoplasmic reticulum.

OLIGOMERIC ORGANIZATION AND METABOLISM

OF THE EPO-R

For several members of the cytokine receptor superfamily, generation of high-affinity receptors requires the formation of hetero-oligomers. Examples include receptors for IL-2, IL-3, IL-5, IL-6, GM-CSF, and CNTF.34-42 The high-affinity receptors for human GM-CSF, IL-3, and IL-5 share a common subunit (called β-subunit),35,36 as do receptors for LIF, IL-6, and CNTF.41,42 While the oligomeric structure of the EPO-R on the cell surface is not yet unequivocally established, various lines of evidence indicate that the EPO-R exists as a multimeric complex.

Cross-linking studies of 125I-EPO to most EPO-responsive cell lines, regardless of ligand affinity, have identified two nonglycosylated or poorly glycosylated polypeptides of molecular weights 100 to 105 Kd and 85 Kd.43 Peptide mapping of these proteins shows that they are similar or identical in structure,44 suggesting that they are derived either by alternative splicing from the same mRNA or by differential proteolytic processing. In COS cells transfected with the EPO-R cDNA, radiolabeled EPO bound to the cell surface can be
cross-linked to polypeptides of apparent molecular weights 65 Kd and ~105 Kd. The 65-Kd polypeptide is almost certainly the product of the cloned EPO-R cDNA; it is immunoprecipitated by antisera specific to the cloned EPO-R, and is immunologically distinct from the 85- and 100-Kd proteins. While the relationship between these polypeptides is not clear, the overall data are consistent with the notion that the 65-Kd EPO-R exists as a multimeric complex with an ~100- to 105-Kd polypeptide.

What is the structure of the high-affinity receptor, and what roles, if any, do the 85- and 100- to 105-Kd polypeptides play in generating high-affinity EPO binding? Correlations between the polypeptides cross-linked to EPO and ligand affinities have produced somewhat disparate results. Initially, D'Andrea et al. reported that expression of the murine EPO-R cDNA in COS cells generates both high-affinity and low-affinity receptors, even though the cDNA was derived from a cell line (MEL clone 745, derived from a mouse infected with SFFV-P) that contains only low-affinity receptors. Others have observed only low-affinity receptors on the surface of transfected COS cells (D. Hilton and S. Watowich, unpublished observations, October 1992). Mutation of Arg-129 to Cys in the exoplasmic domain of the EPO-R (R129C; see below) causes formation of disulfide-linked homodimers, many of which are found on the cell surface, and results in low-affinity receptors that generate an intracellular growth signal in the presence or absence of EPO.

By analogy with two related receptors, additional inferences may be drawn about the oligomeric structure of the EPO-R. The possibility that EPO itself causes formation of noncovalent EPO-R homodimers on the cell surface is supported by analysis of the crystal structure of GH-R. Like EPO, GH is monomeric, and a soluble form of its receptor forms noncovalent homodimers in the presence of a single GH ligand (Fig 2). Is the homodimer sufficient to generate a high-affinity binding site? The G-CSF-R, another related receptor, is able to form a homodimer that is sufficient to generate a high-affinity receptor. The EPO-R appears to have features of both receptors; while the 65-Kd polypeptide can most likely form a homodimer, it is probably not sufficient to generate a high-affinity receptor, as illustrated by the R129C mutation, which generates only a low-affinity receptor complex, at least when expressed in Ba/F3 cells.

Ligand-occupied receptors are internalized by endocytosis, and the bound EPO is degraded in a lysosomal compart-
The intracellular fate of the receptor is only partly understood. Antipeptide antiserum generated against the predicted amino- or carboxy-terminal sequences of the mouse EPO-R immunoprecipitate two forms of the EPO-R from pulse-labeled, EPO-R-expressing heterologous cells (NIH3T3, Ba/F3, HCD57), a nonglycosylated species of molecular weight 62 Kd, and a 64-Kd form that is presumably localized to the endoplasmic reticulum or another premembrane Golgi compartment, displaying endoglycosidaseH (endoH)-sensitive oligosaccharides. Approximately half of the newly made EPO-R is degraded rapidly (t1/2 ~ 40 to 60 minutes) without acquiring endoH-resistance (presumably being degraded in the endoplasmic reticulum), while the rest becomes 66 Kd because of the acquisition of an endoH-resistant oligosaccharide in the Golgi. The 66-Kd species also has a half-life of 40 to 60 minutes, and less than 5% is found on the cell surface. Presumably, the balance of the polypeptide is in vesicles en route from the Golgi to the plasma membrane, lysosomes, or postendocytotic vesicles derived from the plasma membrane. The 66-Kd species is apparently degraded in lysosomes, because inhibitors of lysosome degradation cause accumulation of both intact 66-Kd EPO-R and an endoproteolytic fragment in lysosomes (D. Neumann and H.F. Lodish, unpublished observations, October 1992). These calculations are derived primarily from transfected Ba/F3 cells expressing newly synthesized EPO-R polypeptides, and it is possible that the metabolic fate of the EPO-R may be significantly different in erythroid cells in which putative accessory molecules are expressed. Thus, as analogy to the T-cell receptor complex, the stability of the EPO-R in erythroid cells and its surface expression may be dictated by the presence of accessory subunits. The pp130 is one such candidate.

TRANSMEMBRANE SIGNALING BY THE EPO-R

Given the overall functional similarities among members of the cytokine receptor superfamily, any molecular insight into the mechanism of signaling by one member may be applicable to others. Sequence analysis of the cytoplasmic domains from these receptors has provided no clear understanding of the signal transduction system(s) to which they are coupled. The cytoplasmic domains not only lack homologies to known protein kinases, they also differ greatly in length and show no clear homologies among different members, save for moderate regions of homology between segments of the EPO-R and IL-2-R β-chain. Thus, despite the availability of the cloned receptor, growth-regulatory signals transduced by the EPO-R remain poorly understood. Before cloning of the receptor cDNA, it was shown that EPO induces a rapid Ca2+ influx in erythroid cells infected with Friend virus and in immature erythroblasts. By contrast, mouse CFU-Es did not show a similar increase in intracellular Ca2+. Intracellular cyclic adenosine monophosphate (cAMP) levels increased during EPO-induced signaling as a result of adenylate cyclase activation. Partially conflicting observations were reported in two EPO-responsive cell lines: while EPO induced a rapid increase in cAMP levels and erythroid differentiation in SKT6 cells, it had no effect on cAMP levels in TSA8 cells, even though cAMP potentiated the differentiation of these cells in combination with EPO.
Comparison of the phosphorylation profiles of the wild-type EPO-R with EPO-R mutants having deletions of 30 or 40 residues at the carboxy-terminus shows weak autophosphorylation of the mutant receptors in vitro. As described earlier, the carboxy-terminal region downmodulates proliferation in response to EPO. Phosphorylation of Tyr residues in this region of the EPO-R could negatively regulate intracellular signaling. Alternatively, the carboxy-terminal region could act as a recognition site for a protein kinase that phosphorylates another part of the EPO-R (Figs 1 and 2), as suggested recently for the β-chain of the IL-2-R. This region of the EPO-R may also interact directly or indirectly with neighboring receptors on hematopoietic cells, such as the GM-CSF-R complex, and may be regulated by the same molecule, possibly a novel protein kinase. The auto-phosphorylation sites of Tyr-kinase receptors bind to proteins that contain src homology 2 (SH2) domains. These sequence motifs are found in src protein, other proto-oncogenes, noncatalytic regions of cytoplasmic Tyr-kinases, GTPase activating protein (GAP), phosphoinoside (PI)-kinase, and phospholipase C (PLC)-γ. In several cases, SH2 domains have been shown to bind to Tyr-phosphorylated proteins, including the Tyr-phosphorylated receptor for platelet-derived growth factor (PDGF). We do not know whether the phosphorylated carboxy-terminal region of the EPO-R also binds to an SH2-containing protein.

EPO generates signals for both differentiation and proliferation of erythroid progenitors. This dual action can be dissected by treatment with herbimycin, which selectively inhibits the proliferative activity of EPO. Unfortunately, EPO-induced differentiation has been difficult to analyze at the molecular level, because Ba/F3 and FDCP-1 cells that express transfected EPO-Rs fail to differentiate in response to EPO, even though they become dependent on it for proliferation. Thus, it has not been possible to reconstitute the differentiation response with the cloned EPO-R. The intriguing possibility that additional molecules coupled to the EPO-R may be involved in transducing differentiation signals is suggested by the cross-linking of 125I-EPO to EPO-responsive cells, which shows a unique 119-Kd polypeptide that is not seen in the transfected cells.

PHOSPHORYLATION AND EPO-INDUCED SIGNALING

Protein phosphorylation is an important early step in signal transduction by growth factor receptors, many of which contain intrinsic Tyr kinase domains and exhibit ligand-dependent Tyr phosphorylation. Examples of such receptors include those for PDGF, EGF, CSF-1, and insulin. Several observations suggest that phosphorylation also plays an important role in EPO-R signaling and erythropoiesis. Tyr-kinase inhibitors block the growth of EPO-dependent erythroleukemia cells and induce erythroid differentiation. Murine retroviruses encoding Tyr-kinases such as the abl and src oncogenes efficiently transform erythroid cells. In EPO-dependent cell lines both EPO and IL-3 induce rapid phosphorylation of Tyr residues in several common and several unique cellular proteins. These data suggest the involvement of a protein Tyr-kinase in EPO-mediated signal transduction. However, Ser/Thr phosphorylation has also been implicated in EPO action: inhibitors of protein kinase C block transcriptional induction of c-myc by EPO, an effect presumably mediated by the EPO-R. Inhibitors of Tyr-protein kinases as well as of Ser/Thr protein kinases inhibit the EPO-induced transition of HCD57 cells from the G0 to the S phase of the cell cycle. A regulatory role for Tyr phosphorylation was also suggested. EPO also induced the activity of nuclear protein kinase C in erythroid progenitor cells.

As there is no obvious kinase motif in the cytoplasmic domain of cytokine receptors, attention has been directed toward the identification of cytosolic proteins that may couple ligand-activated receptors to downstream signals. One such protein may be the c-raf proto-oncogene, which encodes Raf-1, a ubiquitously expressed 74-Kd cytosolic Ser/Thr protein kinase. EPO induces rapid phosphorylation of serine and tyrosine residues on Raf-1 in the murine erythroid cell line HCD-57 and in EPO-R-expressing FDC-P1 cells, an effect similar to that seen after stimulation with IL-3, GM-CSF, or IL-2. This is in contrast to the phosphorylation of Raf-1 induced by other classes of mitogens (eg, T-cell activators, PDGF, EGF, insulin, and CSF-1), which occurs predominantly on Ser residues. Finally, antisense oligodeoxyribonucleotides that specifically reduce the intracellular concentration of Raf-1 also inhibit both IL-3- and EPO-stimulated DNA synthesis.

As noted earlier, the EPO-R itself appears to undergo phosphorylation. After EPO stimulation of EPO-R-expressing Ba/F3, UT-7, or DA-3 cells most of the receptor remains in the cell surface. By contrast, this species is virtually undetectable in either NIH-3T3 fibroblast cells expressing the wild-type EPO-R (A. Yoshimura and H.F. Lodish, unpublished observations, July 1992) or in Ba/F3 cells expressing a mutant form of the EPO-R that lacks part of the cytoplasmic domain essential for signal transduction. Thus, formation of the 72-Kd phosphorylated EPO-R species may be closely related to the proliferative action of the receptor. As it might be predicted for a signal-competent molecule, the level of phosphorylated EPO-R decreases to baseline within a few minutes of EPO stimulation. In addition to the 72-Kd species, in vitro phosphorylation of partially purified complexes of EPO and cell-surface EPO-R show an associated 130-Kd protein (pp130), which may be a protein kinase that is copurified with the EPO-R complex or a substrate of the receptor-associated kinase(s) (Fig 2). Anti-phospho-Tyr antibodies precipitate both the 72-Kd EPO-R and the associated pp130, indicating that both molecules contain phospho-Tyr.

In summary, the cumulative data suggest that the EPO-R undergoes phosphorylation in response to EPO, which in turn induces the phosphorylation of other cytoplasmic or membrane proteins. The precise sequence of events that eventually
culminate in a growth signal, as well as the counter-signals that turn off the receptor, remain to be elucidated.

EXPRESSION OF THE EPO-R IN NONERYTHROID CELLS

Murine megakaryocytes and their precursors respond to EPO in serum-free culture; these cells exhibit cell-surface EPO receptors of a single affinity (kd $\sim$ 300 pmol/L). In addition, EPO administration to rodents results in a significant increase in the platelet count and marrow CFU-megakaryocytes (CFU-MK), although the effects are generally more impressive in rodents. These observations strongly suggest the presence of functional EPO-Rs on the surface of megakaryocytes.

In nonhematopoietic cells, binding studies have shown receptors of a single affinity (kd $= 1,000$ pmol/L) in rat and mouse placenta, as well as in embryonic hamster yolk sac and in endothelial cells of human umbilical vein and bovine adrenal capillary. Northern blot analysis of RNA from mouse placenta confirms the presence of EPO-R transcripts (D. Neumann and H.F. Lodish, unpublished observations, October 1992). What is the function, if any, of EPO and EPO-R on these cells? The observation that radiolabeled EPO can be transferred to the fetus from the maternal circulation suggests that the EPO-R may be involved in transcellular transport of EPO, similar to the function of the IgA receptor in transepithelial transport of IgA.

EPO also enhances the migration of endothelial cells. Therefore, quite apart from its function in erythroid cells, the EPO-R may play roles in nonhematopoietic cells that are hitherto unappreciated.

ACTIVATION OF THE EPO-R: MUTATIONS AND INTERACTION WITH A VIRAL PROTEIN

At least three different mechanisms are currently known to trigger the proliferative functions of the EPO-R: (1) binding of its natural ligand, EPO; (2) binding of the env gene product of a murine erythroleukemia retrovirus; and (3) the R129C mutation in the extracellular domain of the EPO-R. The latter two mechanisms, while so far unique to the EPO-R, suggest that leukemogenic viruses or mutations in cytokine receptors play roles in the pathogenesis of many types of leukemia.

Friend virus is a complex of a replication-competent murine leukemia virus (F-MuLV) and a replication-defective spleen focus-forming virus (SFFV). Although this complex is acutely transforming and produces erythroleukemia when injected into adult mice, neither virus in the complex contains oncogenes. The two known strains of SFFV (the anemic and polycythemic strains) differ in the early manifestations of disease, although both ultimately give rise to erythroleukemia. Genetic studies have shown that the virus responsible for induction of erythroleukemia is the defective SFFV, specifically the transmembrane protein product of its env gene, called gp55. The gp55 encoded by SFFV-P binds to the EPO-R and activates it for proliferation, resulting in growth of the host cells irrespective of the presence of EPO. While a large portion of the gp55–EPO-R complex accumulates in the endoplasmic reticulum, some of it is expressed on the cell surface; this fraction is thought to be essential for the transforming ability of SFFV. The binding of gp55 does not interfere with the binding of EPO to the EPO-R, implying that these “ligands” bind to different sites on the extracellular domain of the EPO-R. It is principally the transmembrane domain of gp55 that confers EPO-independent cell growth. When chimeras of the EPO-R and the IL-3-R polypeptides were expressed in Ba/F3 cells, only those molecules that contained the transmembrane domain of the EPO-R could be activated for proliferation by gp55, thereby emphasizing the interaction of gp55 and EPO-R via their transmembrane domains.

Considerably less is known about the mechanism by which the SFFV-A strain of Friend virus causes leukemia. Leukemic cell lines isolated from animals infected with SFFV-A remain dependent on EPO for growth, and despite much effort it has not been possible to demonstrate functional or physical interactions between the EPO-R and the env gene product of SFFV-A. The nucleotide sequences of env genes from the “A” strains have been compared with their SFFV-P counterparts to identify potentially important differences. Typically, env genes of SFFV are the products of recombinations and deletions of their counterpart genes from other retroviruses: the amino terminus of gp55 molecules are derived from the env genes of endogenous mink cell focus-inducing (MCF) viruses and the carboxy termini from ecotropic F-MuLV env-like sequences. The greatest sequence disparity between the env genes of SFFV-P and SFFV-A resides in and around the transmembrane domain. Chimeras of the env genes of the SFFV-A and SFFV-P viruses indicate that the $\sim 40$ amino acids, including the transmembrane region and carboxy-terminus, determine the ability of the env gene to stimulate EPO-independent proliferation. These observations suggest that the transmembrane region of SFFV is important for activation of the EPO-R. The env protein of the MCF virus can also bind to and activate the EPO-R, and possibly the IL-2R, through interaction of its amino-terminal domain with the EPO-R. Lastly, the replication-competent helper virus F-MuLV can also produce leukemias of multiple lineages in newborn mice, but not in adults, suggesting that erythroleukemias induced by F-MuLV result from the generation of MCF-like env genes in adult mice.

Two classes of mutations that enhance the activity of the EPO-R have been identified by a genetic selection strategy that takes advantage of the inherent mutagenic potential of retroviral transduction systems. The first class (tEPO-R) includes the carboxy-terminal deletions noted earlier. The cDNA of tEPO-R appears to have sustained a deletion spanning the 3' coding and noncoding region, resulting in the replacement of the terminal 42 amino acids (from Gly 1424 to Gly 1616) of the wild-type EPO-R with two amino acids, Ala and Leu. This mutation renders the receptor hypersensitive to bound EPO allowing cells expressing tEPO-R to grow in 1 pmol/L EPO, one tenth the physiologic concentration, but not in its absence. Otherwise, as stated earlier, Ba/F3 cells expressing tEPO-R and wild-type EPO-R have similar numbers of cell-surface EPO-Rs, similar binding affinities for EPO, and similar characteristics of endocytosis and degradation of bound EPO. The second class of mutations, termed constitutive (cEPO-R), confers on the cell the ability to grow autonomously in the absence of EPO or other growth factors. Two different mutant cEPO-Rs have been identified.
contains a single C to T substitution in an otherwise wild-type cDNA, resulting in the substitution of a Cys for Arg at codon 129 in the exoplasmic domain (EPO-R R129C). Another is a double mutant containing the carboxy-terminal deletion of the EPO-R as well as the R129C mutation.

The physiologic effects of the R129C mutation mimic precisely the activation of the wild-type receptor by gp55. In addition to conferring factor-independent growth, the mutation causes accumulation of the receptor in the endoplasmic reticulum, and prevents the rapid intracellular degradation characteristic of the wild-type receptor. As noted above, the R129C mutant exists as disulfide-linked dimers on the cell surface as well as in the endoplasmic reticulum, an effect directly attributable to the mutant Cys at codon 129. Thus, although there is no direct evidence that EPO induces dimerization of the cell-surface receptor (Fig 2), the disulfide-linked R129C homo-dimer is thought to mimic dimerization of the wild-type EPO-R triggered by EPO binding.

To determine whether the R129C mutant could contribute to the development of leukemia, a recombinant SFFV was generated in which the gp55 was replaced with the R129C EPO-R. When injected into adult mice, the virus caused polycythemia and splenomegaly. Clonal, growth factor-independent erythroid cell lines were isolated from infected mice expressing the R129C EPO-R. Injection of these cells back into mice resulted in rapid development of erythroleukemia. This was the first demonstration of a truly oncogenic point mutation in a member of the cytokine receptor superfamily.

Unlike infection with wild-type SFFV, infection with recombinant SFFV expressing the R129C EPO-R showed a delay in the appearance of early polycyetal erythrocytosis (1 to 2 weeks v 5 weeks); a large increase in the number of megakaryocytes in the spleen, comparable with wild-type SFFV, but also associated with a transient increase in circulating platelet numbers; and expression of the R129C EPO-R in splenic megakaryocytes (Longmore G, Neumann D, Lodish HF; submitted). Infection of fetal liver cells with this mutant SFFV also causes proliferation of CFU-E, BFU-E, and CFU-GM cells. However, there is no obvious arrest in differentiation of these cells due to expression of the R129C EPO-R. These results demonstrate that SFFV is capable of infecting and expressing its genes in cells of multiple hematopoietic lineages. Why, then, is SFFV-induced disease by viruses expressing the R129C EPO-R restricted to erythroid cells?

Early in the course of SFFV virus infection there is a polyclonal erythroblastosis, without any increase in the self-renewal capacity or abrogation of differentiation potential of the cell. After 4 to 6 weeks clonal, growth factor-independent, leukemogenic cells evolve, which are arrested at the proerythroblast stage of differentiation. Activation of the EPO-R by gp55 is thought to be responsible for the early erythroblastosis, which could facilitate accumulation of subsequent genetic mutations that result in the promotion of a leukemic clone altered in its differentiation program. Similarly, the structurally activated EPO-R R129C could supplant gp55 as an oncogenic agent in Friend virus-induced erythroleukemia by promoting uncontrolled erythroblastosis, resulting in the acquisition of further genetic changes that culminate into frank leukemia. As with gp55, the role of EPO-R R129C, if any, in the later stages of the disease are unclear.

Later events thought to contribute to the development of immortal, leukemic cells in SFFV-induced erythroleukemia include insertional activation of the putative oncogene sp1, and/or inactivation of the suppressor oncogene p53. Assuming that many types of mutations are acquired during active DNA replication, the expression of EPO-R R129C in nonerythroid cells may not induce a sufficient degree of cell proliferation to allow accumulation of secondary mutations necessary for leukemogenesis. Alternatively, distinct batteries of secondary mutations may be required for the development of tumors derived from different types of hematopoietic progenitors.

Another major function of EPO is the prevention of programmed cell death (apoptosis) in late-stage erythroid progenitors. An alternatively spliced form of the human EPO-R that causes truncation of the cytoplasmic domain was found to be the most abundant mRNA species in early erythroid progenitors, whereas the full-length species was found to predominate in late progenitors. Although the truncated receptor transduced a mitogenic signal, it was significantly less competent than the full-length receptor in preventing apoptosis of transfected cells. These findings suggest that stage-specific posttranscriptional changes in the expression of the EPO-R can give rise to receptors with distinct functional roles.

GENETIC LOCUS OF THE EPO-R

Soon after the cloning of the cDNAs for the mouse EPO-R, the complete genomic sequences encoding the EPO-R and the regulatory elements that govern its transcription were characterized. More recently, genomic sequences of the human homologue of the murine receptor and those of two other members of the cytokine receptor superfamily have also been determined. Several important observations have emerged from these studies.

Chromosomal localization and Southern analysis showed that both the mouse and human EPO-R genes are present in a single copy per haploid genome. The gene for the mouse EPO-R is on the proximal arm of chromosome 9, near the centromere and closely linked to the low-density lipoprotein receptor locus, while the human gene is on chromosome 19p. These assignments do not show any obvious linkages to pathologic disorders of erythropoiesis. Furthermore, unlike the impressive clustering of other growth factor genes and their receptors on human chromosome 5q, there is no similar clustering of members of this family of receptors or their ligands. For example, the gene for the human IL-2-R β-chain is on chromosome 22, 14, 15 the IL-4-R gene is on human chromosome 16 and mouse chromosome 7, and the mouse IL-5-R gene on chromosome 6. The chromosomal locations of the ligands are also widely dispersed (eg, the gene for human EPO is on chromosome 7). Although the genes for IL-3 and GM-CSF are syntenic (chromosome 5q), those for their receptors as well as those for other members of the cytokine superfamly are not syntenic.

Despite the lack of chromosomal clustering, a remarkable conservation exists at the level of genomic organization of
These receptors. Both the mouse and human EPO-R genes are relatively small, spanning 5 to 6.5 kb, with 8 exons and 7 introns. The sizes of individual exons and introns and the intron–exon junctions show an extremely high degree of conservation. The overall identity of the nucleotide sequence of the coding portions of the mouse and human genes is 81.6%. Another region of homology is in the upstream sequences neighboring the transcriptional start sites. In the 3′ region of the mRNA, the homology ends abruptly after the stop codon. Therefore, it appears that this relatively small locus defines a complete transcriptional unit (Fig 3).

The conservation of genomic structure also extends to two other genes of this receptor superfamily characterized recently, those for the human IL-2-R β-chain and the human and mouse IL-7-R genes. Like the EPO-R gene, the IL-7-R gene also contains 8 exons and 7 introns. However, the IL-2-R β-chain gene has 10 exons: the first encodes 5′ untranslated sequences, and the fourth and fifth exons together correspond to the third exon of the EPO-R. The grouping of these two exons into one facilitates the alignment of the coding regions of these receptors and leads to the following observations. First, the extracellular domain of these receptors is encoded by five exons, except for the human IL-2-R β-chain gene which contains the “split” exon. Second, each pair of conserved Cys residues is encoded by a separate exon. Third, the transmembrane domain is encoded by a single exon. This organization sequesters the exon encoding the WSXWS motif from that encoding the transmembrane domain. Fourth, the cytoplasmic domain of each receptor is encoded by two exons, a small exon encoding the membrane-proximal region, and a much longer one encoding the bulk of the cytoplasmic domain as well as all of the 3′ noncoding sequence. These observations clearly show the evolutionary relationship of genes encoding these cytokine receptors, and suggest the intriguing possibility of an ancestral gene that gave rise to some, if not all, members of the cytokine receptor superfamily.

**Expression of the EPO-R Gene**

Inasmuch as the expression of the EPO-R by hematopoietic cells represents one of the earliest events after commitment to the erythroid lineage, identification of its regulatory elements could shed light on the molecular events of early erythropoiesis. Transcriptional activation of the EPO-R gene occurs when murine embryonic stem cells differentiate in vitro into hematopoietic precursors. However, the specific erythropoietic stage at which this initial gene activation takes place is unknown. As described earlier, certain cultured erythropoietic cells exhibit a dramatic increase in the number of cell-surface EPO-Rs upon progression from the BFU-E to the CFU-E stage. This second increase in the level of the EPO-R is at least partly the result of an increase in transcription of the EPO-R gene. Additional evidence for such a second transcriptional activation step comes from a comparison of two murine cell lines arrested at different stages of erythropoiesis. CB5 cells are thought to be arrested at the BFU-E stage of erythropoiesis. By Northern analysis, EPO-R expression in these cells is about one fifth to one tenth the level in the more mature MEL cells (H. Youssoufian, submitted). Hence, the transcription of the EPO-R gene, in addition to being tissue specific, is also temporally regulated during erythropoiesis.

What are the cis- and trans-acting elements responsible for these regulatory events? Transcriptional initiation of both the mouse and human EPO-R genes occurs in the 5′ flanking region about 150 bp upstream of the ATG initiation codon. In the vicinity of the transcription initiation sites there are no TATA or CAAT boxes characteristic of many tissue-specific genes. However, there are potential binding sites for the ubiquitous Sp-1 and the erythroid-specific GATA-1 transcription factors (Fig 3). The location of these elements is well conserved relative to the transcription initiation sites in the mouse and human genes. It is possible that GATA-1 is involved in transcriptional activation and
transcription initiation site determination, possibly through association with TFIIID or other transcription factors, and in erythroid-specific genes a functional GATA-1 binding site may supplant the more usual TATA element. A 452-bp fragment derived from the segment immediately 5' of the transcription initiation site of the mouse EPO-R gene directs erythroid-specific transcription of a linked reporter gene. This region includes the Sp-1 and GATA-1 binding sites, both of which bind their cognate proteins. Single-base mutations at either site greatly diminish the promoter activity of this fragment. Mutations in a CACCC site about 40 bp upstream of the GATA-1 binding site also greatly diminish promoter activity (H. Youssoufian, unpublished observations, June 1992). Thus, the minimum promoter for the mouse EPO-R gene includes binding sites for Sp-1, GATA-1, and the CACCC-binding protein (Fig 3). Furthermore, the EPO-R promoter is transactivated by coinexpression of GATA-1 in heterologous cells (NIH3T3, COS) that ordinarily express neither gene. Signals generated by the EPO-R may, in turn, enhance the expression of the GATA-1 gene, creating a positive feedback loop. These observations point to the central contribution of the erythroid-specific GATA-1 transcription factor to EPO-R promoter activity.

Biochemical studies of EPO-induced terminal differentiation of CFU-Es and proerythroblasts have identified a set of erythroid proteins that are induced (eg, band 3 and band 4.1) or enhanced (eg, spectrin) by exposure to EPO. Considering that GATA-1 may also activate the transcription of its own gene, these studies, taken together, begin to define some of the constituents of the genetic activation cascade of mammalian erythropoiesis (Fig 4). The dual positive feedback loops that culminate in the activation of the EPO-R gene may help to reinforce the sequential activation of genetic information along the pathway of erythroid differentiation, and thus maintain the erythroid-committed state.

Although the activation of the "minimum" 452-bp promoter may account for the "basal" level of transcription that is seen at the BFU-E stage, it probably cannot account for the second activation phase during differentiation to the CFU-E stage. The contribution of other potential regulatory sequences to the activity of the EPO-R promoter is now being studied. Sequences further upstream of the minimum promoter seem to have both positive and negative regulatory functions. The latter includes a transcriptionally active repetitive element that interferes with the activation of transcription by the downstream EPO-R promoter. The in vivo role of this element, if any, is not known. In both erythroid and nonerythroid cells, there are DNase I hypersensitive sites in the 5' flanking region (H. Youssoufian, unpublished observations, June 1992) and in intron 1 of the EPO-R gene, some of which appear to be erythroid specific (Fig 3). Such sites are the hallmarks of "open" chromatin, and are thought to make the neighboring genes and promoter elements more accessible to transcription factors. One of these sites (site D, Fig 3) within intron 1 appears to have enhancer activity. Another site (site A, Fig 3) is associated with stage-specific enhancer activity, because both the hypersensitive site and the associated enhancer activity are present in MEL cells but not in CB5 cells (H. Youssoufian, unpublished observations, June 1992). Others have not discerned stage-specific differences in DNase I-hypersensitive sites of the EPO-R gene. Further analysis of these elements should yield a reasonably complete picture of the transcriptional activation of this receptor.

Finally, while it is clear that GATA-1 is necessary for the activation of the EPO-R promoter, its expression in concert with constitutive transcription factors may not be sufficient to activate the EPO-R gene (H. Youssoufian and G. Longmore, unpublished observations, July 1992). A preliminary survey of a number of hematopoietic cell lines, both erythroid and nonerythroid, by Northern analysis shows some discordance in the expression of GATA-1 and EPO-R. It is possible that in some cells the level of GATA-1 protein is below the threshold required to activate the EPO-R gene. It is also possible that additional transcription factors (eg, SCL) may be required to activate the EPO-R locus. Alternatively, the EPO-R gene may be activated in certain cell types by GATA-1-independent mechanisms.

THE EPO-R AND HUMAN DISEASE?

The finding that the EPO-R can be activated for proliferation by a single point mutation, partial deletion, or by insertional activation of the EPO-R by the LTR of SFFV in murine systems has stimulated the search for genetic defects in the EPO-R in human hematopoietic disorders. To date, one such defect has been described, a 3' end deletion of an EPO-R gene in the human erythroleukemia cell line TF-1. The cell line overexpresses EPO-R mRNA, which otherwise appears to be structurally normal. This finding is consistent with the animal leukemia model described earlier, whereby excessive activity of the EPO-R leads to leukemogenesis. In another study, multiple transcripts of the EPO-R were identified in a human erythroleukemic cell line, although the precise genetic defect responsible for this phenomenon is not known.

A candidate disorder for EPO-R defects is polycythemia vera (PV), a clonal myeloproliferative disorder that presents primarily with erythrocytosis, although elevations in granulocytes and platelets also occur. The circulating serum levels of EPO are lower than normal or normal, and ex vivo cultures of erythroid progenitors derived from patients with PV grow and differentiate independently of EPO. Such cells may also be hypersensitive to EPO or to other growth factors such as IL-3 or insulin-like growth factor 1. As
observed in MEL cells, CFU-Es from patients with PV have a single class of low-affinity EPO-Rs (kd = 720 pmol/L), and cross-linking experiments have identified surface proteins similar in size (90 and 100 Kd) to those of normal erythroid progenitors. This situation is reminiscent of murine model of EPO-R activation by either the R129C mutation or interaction of the wild-type EPO-R with gp55. The effect of an activated EPO-R need not be restricted to erythroid cells; its expression in megakaryocytes may provide an explanation for the associated thrombocytosis (Longmore G, Neumann D, Lodish HF; submitted). The implication of a hyperfunctioning EPO-R as the etiology of granulocytosis is more difficult to justify; EPO-R may have a subtle effect on CFU-GM growth, or it may be expressed ectopically in the context of a neoplastic cell. It must be emphasized that these points are purely speculative, but appropriate molecular diagnostic techniques should help resolve the role of EPO-R in this disorder.

Another candidate disorder is familial erythrocytosis, a heterogeneous group of hereditary conditions manifested by increases in RBC volume. Many of these conditions are caused by mutant hemoglobin molecules that have an increased affinity for oxygen. However, in a few families the disorder appears to be intrinsic to erythroid precursors that are hypersensitive to EPO. This observation suggests possible alterations in the signal transduction pathway(s) of the EPO-R. However, analysis of the number, affinity, and gross molecular structure of the EPO-R in three families afflicted with this disorder failed to show any differences from the wild-type EPO-R. The possibility that there are specific point mutations that constitutively activate the EPO-R remains to be tested (J.T. Prchal, personal communication, June 1992).

Diamond-Blackfan anemia (DBA) is a congenital RBC aplasia with severe normochromic-macrocytic anemia. The BM is deficient in erythroid precursors that appear to be arrested in differentiation; other hematopoietic lineages are normal. Serum EPO levels in DBA tend to be elevated, and BM CFU-E and BFU-E growth ex vivo is defec-tive. These observations suggest that EPO-induced growth of erythroid progenitors, presumably via the EPO-R, is defective in DBA. The growth of CD34+ cells, in vitro, from the marrow of DBA patients suggests that the defect lies in the inability of multipotent progenitors (CFU-GEMM) to undergo erythroid differentiation. The addition of SCF to EPO and IL-3 can overcome this block, and clinical trials with IL-3 appear to ameliorate the disease. There are no abnormalities in the structure or function of c-kit or its ligand SCF. These data suggest defects in EPO-R structure or signaling. Molecular studies of the EPO-R may shed light on the pathogenesis of this disorder.

Finally, in a number of disorders characterized by high levels of circulating EPO, such as RBC aplasia, in vitro erythropoiesis is decreased, and erythroid progenitors are poorly responsive to exogenous EPO. Abnormalities in EPO binding or EPO-signaling remain to be elucidated.

FUTURE PROSPECTS

Molecular elucidation of the structure and function of the EPO-R is vital to our understanding of mammalian erythropoiesis. In addition to providing us with a more profound view of this hematopoietic lineage, such insights also can facilitate the extension of basic knowledge to clinical circumstances. For example, ligand-receptor interactions are now attractive targets of therapeutic intervention. The design of compounds that mimic the action of EPO or, conversely, block excessive activity of the EPO-R in some forms of leukemia or myeloproliferative disorders may provide clinicians with powerful therapeutic tools. The structural subunits required to generate a high-affinity receptor are likely to be solved in the near future. At the level of transcriptional activation, the interactions of GATA-1 or other transcriptional factors with the EPO-R promoter should improve our understanding of erythroid-specific gene regulation. Finally, the mechanism of signal transduction by the EPO-R continues to be dissected. It seems plausible that several members of the cytokine receptor family share a common pathway for intracellular signaling, perhaps involving noncovalent associations of the same or closely related Tyr kinases with the cytoplasmic domains of these receptors. These molecules are likely to mediate fundamental activities involved in cell growth, differentiation, and apoptosis. The eventual identification of such molecules and characterization of their natural substrates should significantly advance our understanding of the molecular regulation of hematopoiesis.

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