Erythropoietin Receptor

Subunit Structure and Activation

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Physiologic role of erythropoietin in erythroid differentiation

Erythropoietin (EPO)[1] is a 34-kD glycoprotein hormone which is the primary regulator of human erythropoiesis. A cDNA encoding EPO has been isolated (1), and recombinant EPO with oligosaccharide moieties identical to the natural material is available (2). EPO is synthesized and released by the kidney, and it circulates to the bone marrow where it stimulates resident erythroid progenitors via a specific receptor (3). EPO as a pharmacologic agent has clearly demonstrated efficacy in anemic states such as kidney failure (4, 5). In vivo, EPO causes a rapid rise in hematocrit in 7–10 d.

Despite the availability of purified recombinant EPO, little is known regarding the interaction of EPO and the erythropoietin receptor (EPO-R) or the physiologic mechanisms by which EPO causes cells to undergo proliferation or differentiation. This is largely due to the lack of adequate quantities of EPO-R for in-depth biochemical study. Only small numbers of surface EPO-R (< 1,000) are present on normal erythroblasts and erythroleukemia cells. EPO provides a proliferative signal to the Burst-forming unit-erythroid (BFU-E), an early EPO-responsive erythroid progenitor, and a differentiative signal to the colony-forming unit-erythroid (CFU-E), a later EPO-responsive erythroid progenitor (3). These two classes of EPO-responsive cells are clearly different populations since they can be separated by unit gravity sedimentation and more recently have been purified to homogeneity (6). The purpose of this review is to describe the recent advances in our understanding of EPO-R physiology, derived from the cloning of the EPO-R cDNA.

Structure of the EPO-R

Before the cloning of the EPO-R cDNA, investigators have used radiolabeled EPO to demonstrate specific binding to cells derived from the erythroid lineage. These cells include normal erythroid progenitors, virally transformed spleen cells (i.e., Friend cells), murine and human erythroleukemia cells, and cells from human fetal liver. Since there is 80% protein homology between the murine and human EPO polypeptide, most of the studies described used iodinated recombinant human EPO. Scatchard analysis has been employed to determine the number of binding sites per cell and the affinity constants for the interaction. As shown in Table I (references 6–37), ~ 200 EPO-R are present on the cell surface of purified normal erythroid progenitors. On certain cell lines, that number can increase to about 1,000 per cell. This relatively low number of EPO-R is characteristic of other receptors for hematopoietic cytokines such as G-CSF, GM-CSF, IL-3, and IL-6 (reviewed in references 38–40). Scatchard analysis revealed that certain erythroid cells, such as MEL cells, express only low-affinity receptors whereas other cell lines express high- and low-affinity receptors (Table I). Of note, the lower-affinity receptor has an affinity constant in the 200 pM range and therefore still represents an intimate interaction between receptor and ligand. Although functional differences between the higher- and lower-affinity EPO-R have not been determined, the two affinities may account for different cellular responses to EPO. Friend virus-infected cells respond to EPO with proliferation and differentiation. MEL cells bind EPO only with low affinity and do not appear to respond to the hormone. Affinity cross-linking experiments using radiolabeled EPO (Table I) reveal two cross-linked EPO-R complexes. The complexes appear either as two bands of sizes 145 and 120 kD or as one band of 110 kD. The size of the band is the sum of the size of EPO (34 kD) and the size of the receptor. Other higher- and lower-molecular-weight species have been documented. There is also disagreement on the effect of reducing agent on the cross-linking experiments. Some investigators have demonstrated one large molecular species of ~250 kD which resolves to two bands when reduced (20), while others have not demonstrated this high-molecular-weight band (19). A relationship between the number of bands and the presence of high- and low-affinity receptors is not evident.

The recent cloning of the murine EPO-R (41) has provided new insights into the understanding of EPO-induced signal transduction. The EPO-R cDNA was cloned by transfecting pools of recombinant plasmids from a MEL cDNA library into COS cells and screening for uptake of radioiodinated EPO by transfected cells. A single cDNA was isolated which was capable of conferring on COS cells the ability to bind EPO. As inferred from the sequence of the cDNA, the cloned EPO-R is a 507-amino acid polypeptide with a single membrane-spanning domain, or a so-called type I membrane-spanning protein (Fig. 1). As expected, the EPO-R transcript showed erythroid specific expression (41). Surprisingly, the COS cell transfectants, unlike MEL cells, demonstrated both high- and low-affinity receptors (Fig. 2 *A*). This result suggested that the cDNA encoded one subunit of the EPO-R, and that a second subunit, or accessory protein, was endogenous to COS cells. The COS cell transfectants also demonstrated two cross-linked com-

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1. *Abbreviations used in this paper:* EPO, erythropoietin; EPO-R, erythropoietin receptor; PV, polycythemia vera; SFFV, Friend spleen focus-forming virus.

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Table I. Expression of the Erythropoietin Receptor: A Literature Review

Cells or cell lines	Total receptor number	Affinity	Receptor size by affinity crosslinking	Reference No.
		nM	*kD*	
Murine cell lines: erythropoietin-independent				
MEL 745	110–910	0.1–3.3	100, 85 or 63–76	7, 8, 9, 10
GM979	110–1600	0.15–0.67	63	9, 11
707	120	0.78	63	9
B8	350–650	1.2–1.4	109, 94	12
K-1	930	0.28	63	9
Murine cell lines: erythropoietin-responsive				
TSA8	90–680	0.1–0.3, 6.1	106, 86	8, 13
SKT6	470	0.15	119, 94, 63	14
Rauscher red 1	164	2.7		10
Rauscher red 5-1.5	1,200–1,700	0.44	105, 95	11, 15
J2E-1	1,035	0.279		16
Murine cell lines: IL-3–dependent				
DA-1	20–131	0.54–1.4		17, 18
FDC-P2	20–60	0.8–1.4		18
IC-2	20–60 or none	0.8–1.4		9, 18
Murine cells				
Friend cells	180–1,000	0.08–0.09 (20%), 0.57–0.60 (80%)	100, 85	8, 19, 20, 21, 22
Bone marrow cells			102, 85	20
Bone marrow cells (rat)	38	0.18	75	23
Fetal liver (rat)	487	0.167	94, 78	24
Regenerating erythroblasts	43–62	0.09		23
Phenylhydrazine-treated spleen cells	30–450	0.09–1.7		25, 26
CFU-E	370	0.073 (40%), 0.813 (60%)		6
Fetal liver	402–1,220	0.1–0.41 (75%), 3.13 (25%)	101, 96, 56, or 106, 86	8, 13, 20, 27
Yolk sac erythroid cells (hamster)	30–722	0.01–0.376		28, 29
Megakaryocytes (mouse and rat)	6,500	0.287		30
Human cell lines: erythropoietin-unresponsive				
K562	4–6 or none	0.270–0.290		7, 9, 31
HEL	31–34 or none	0.128–0.293		9, 32
OCI-M1	3,000	0.28		11
Human cell lines: erythropoietin-responsive				
JK-1	310	0.06 (35%), 0.4 (65%)	106, 86	33
KU812	205	0.25		34
TF-1	1,630	0.4	105, 90	35
Human cells				
CFU-E	1,050	0.1 (20%), 0.57 (80%)	100, 85	36, 37
Placenta		0.6–1.4	100, 85	36

The cells or cell lines listed have been examined for EPO-R expression using radiolabeled ^{125}I-EPO in standard binding conditions. The total receptor number and affinity were determined by Scatchard analysis. If two distinct affinities were determined, both affinity constants and the relative percentages of each affinity is listed. Affinity cross-linking studies used radiolabeled ^{125}I-EPO for binding and disuccinimidyl suberate (in general) for cross-linking. The products were assayed in the presence of reducing agent.

plexes (Fig. 2 *B*). These cross-linked complexes were similar in molecular weight to those observed in normal erythroid progenitor cells (Table I).

A new growth factor receptor superfamily

The EPO-R was initially found to have extensive amino acid homology with the interleukin 2 receptor β chain (42). The IL-2 receptor is an α/β heterodimeric receptor with two receptor affinities (43, 44). Interestingly, the EPO-R cDNA and the IL-2R cDNA each generate high- and low-affinity receptors and two radiolabeled cross-linked polypeptides when expressed in stable transfectants (42). The native EPO-R, therefore, probably contains a second chain, and a second chain may be endogenous to COS cells. Also, the greatest sequence identity (35% amino acid identity) between the EPO-R and the IL-2R β chain exists in a cytoplasmic region, adjacent to the transmembrane domain (Fig. 3). Since no tyrosine kinase catalytic domain is evident, this conserved region is of particular importance and probably denotes common signal transduction mechanisms. Interestingly, both EPO-dependent (45) and

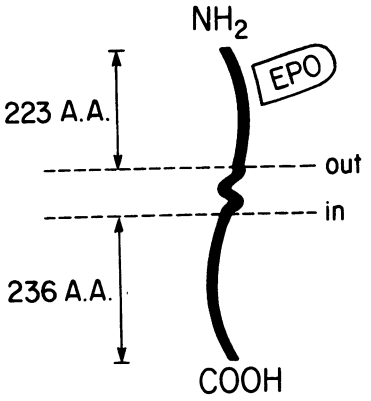

Figure 1. Schematic model of the murine erythropoietin receptor, EPO-R. The predicted sequence of the EPO-R (41) is 507 amino acids. Cleavage of a 24-amino acid hydrophobic leader sequence leaves a 223-amino acid extracytoplasmic domain which contains the EPO binding domain. The 236-amino acid cytoplasmic tail contains the signal transduction domain.

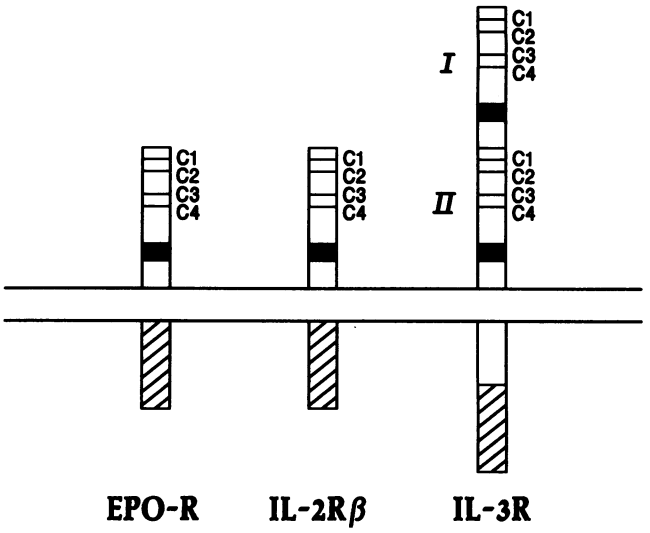

Figure 3. A new cytokine receptor superfamily. Homologous domains of the cytokine receptors EPO-R (41), IL-2R β (42), and IL-3R (46) are aligned. The extracellular domains share a highly similar 20-amino acid sequence which includes a conserved WS-WS motif (*black box*), presumed to be involved in protein–protein interaction. Also, four conserved cysteine residues (*C1–C4*) are aligned. The homologous external domain is duplicated in the IL-3R (regions I and II). The cytoplasmic domain of these three receptors contains a conserved region, rich in proline, serine, and acidic residues (cross-hatched bars), suggesting a common mechanism of signal transduction.

IL-2-dependent (43) cell lines have been characterized, suggesting that cell viability and proliferation is conferred by these hormones.

The EPO-R family of receptors, or so-called cytokine receptor superfamily, has now expanded to include several other growth factor receptors. These include the IL-3 receptor (46), the IL-4 receptor (47, 48), the IL-6 receptor (49), the GM-CSF receptor (50), and the prolactin and growth hormone receptor (51). The most striking similarity among all members of this cytokine receptor superfamily includes the conservation of four cysteines and a tryptophan-serine-X-tryptophan-serine motif positioned just outside the transmembrane region. These sequence homologies probably reflect structural homologies among these receptors. The receptor structural homologies may extend to structural homologies among the respective growth factors themselves. The crystal structure of growth hormone reveals an antiparallel four-helix bundle core. Modeling of the tertiary structure of EPO and IL-6 also reveals a growth hormone-like helix bundle fold (J. F. Bazan, personal communication). There also exists extensive amino acid identity among the EPO-R, IL-2R, and IL-3R in the cytoplasmic domain, suggesting common signaling mechanisms (Fig. 3). It is likely that all members of this family are multi-subunit re-

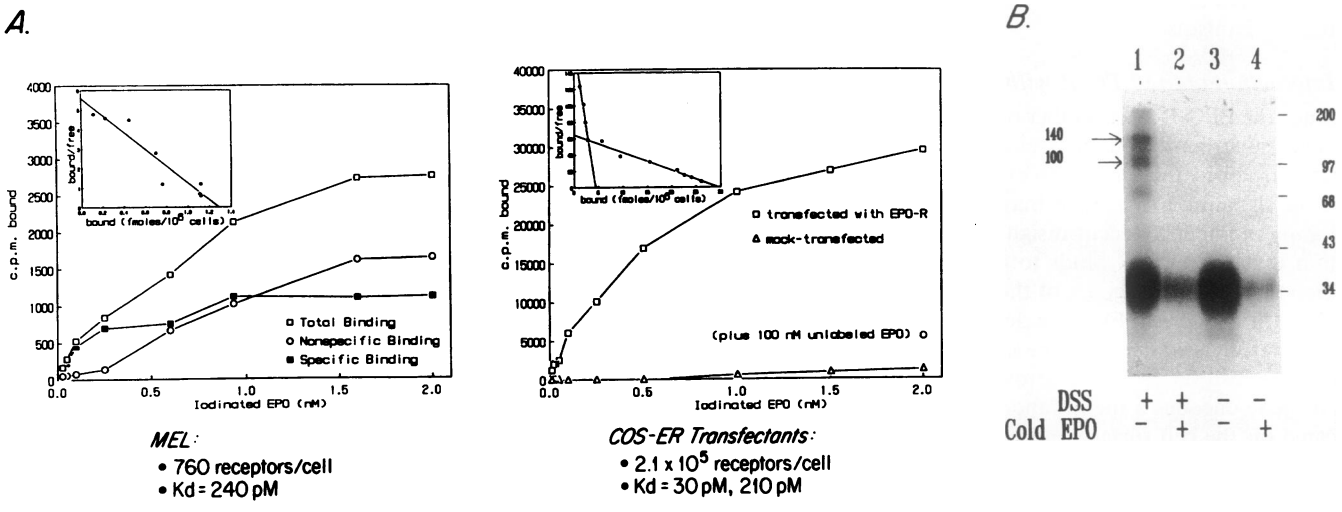

Figure 2. Binding characteristics of the murine EPO-R. (*A*) Comparison of recombinant EPO binding to MEL cells and COS cells expressing recombinant EPO-R. MEL cells grown on fibronectin monolayers or COS-EPO-R transfectants were incubated with various concentrations (*x*-axis) of radioiodinated recombinant human EPO at 4°C for 8 h. For MEL cells, nonspecific binding was determined by coincubation with 100 nM of unlabeled EPO. For COS cells, nonspecific binding was determined by incubating EPO with COS cell monolayers mock-transfected with the PXM vector. Insets show Scatchard analyses of the same data. (*B*) Cross-linking of radiolabeled EPO to EPO-R expressed in COS cells. Cross-linking procedure has been previously described (41). In the presence of the cross-linking agent, DSS disuccinimidyl suberate (DSS) and in the absence of cold EPO (lane *1*), two major cross-linked complexes are observed of 140 and 100 kD. When excess EPO is added (lane *2*) or when DSS is omitted (lanes *3* and *4*), the cross-linked complexes are not seen.

ceptors and that additional subunits account for the discrete hormone affinities observed.

Transmembrane signaling by the EPO-R

Several studies have investigated the cellular action of the erythropoietin ligand–receptor complex. Using the intracellular calcium chelators, quin-2 and fura-2, EPO has been shown to induce a rapid increase in intracellular free calcium (52, 53). EPO has also been demonstrated to activate Mg^{2+}, Ca^{2+}, and Na^{+}-K^{+} ATPases. Monesin, a sodium specific ionophore, potentiates erythroid growth, whereas valinomycin, a potassium-specific ionophore, suppresses growth. Investigators have reported that EPO activates adenylate cyclase and increases cyclic AMP levels, independent of activation by β-adrenergic receptors (54, 55). Conflicting results have been found concerning the role of guanidine nucleotide-binding proteins in EPO stimulation. Phospholipase C and phosphoinositol mechanisms do not appear to mediate the noted calcium flux. Also, Choi et al. (56) have described a protein of 43 kD which is dephosphorylated on serine residues in erythroid cell lines in response to EPO.

As discussed previously, the major difficulty encountered in studies of EPO-induced signal transduction has been the small number of surface receptors and the unavailability of a purely EPO-dependent cell line. The EPO-R, although expressed in high numbers in COS cell transfectants (41), is not functional in these cells. The addition of EPO to these transfectants does not change the growth kinetics of the cells or induce erythroid differentiation (D'Andrea, A., unpublished observation). In contrast, transfection of the EPO-R cDNA into Ba/F3 cells, a murine cell line dependent on the hormone, interleukin 3 (multi-CSF), does confer EPO dependence (57, 58). An EPO-dependent clone of Ba/F3 cells (Ba/F3-EPO-R) has been isolated which expresses cell surface EPO-R and which has absolute dependence on EPO for growth. The availability of this homogeneous, EPO-dependent cell line should greatly facilitate the study of the EPO-R structure and signaling mechanisms.

Interaction of the EPO-R with the Friend virus gp55

Since the EPO-R is a member of a large hematopoietic growth factor superfamily, any molecular insight into the EPO-R subunit structure, the generation of its multiple affinities for EPO, or its signaling mechanism may be generalizable to the other family members. Recent insight is derived from the observation that the EPO-R binds to and is activated by the membrane glycoprotein, gp55, of the Friend spleen focus-forming virus (SFFV) (58). SFFV is a defective murine C-type retrovirus that causes a multistage erythroleukemia in mice and erythroblastosis in bone marrow cultures (59, 60). The SFFV *env* gene encodes a membrane glycoprotein, gp55, that is located on the cell surface and within rough endoplasmic reticula (61) and that is essential for the induction of leukemia in vivo (62) and the erythropoietin-independent erythroblast proliferation in vitro (63). By co-transfecting both EPO-R and the gp55 into an interleukin-3–dependent lymphocyte cell line, it was shown that the physical interaction of these two proteins gives rise to autocrine cell growth. The interaction between the EPO-R and gp55 is shown schematically in Fig. 4. The EPO-R is a 507-amino acid, type I membrane-spanning protein. The gp55 is 409 amino acids, with a carboxy-terminal membrane

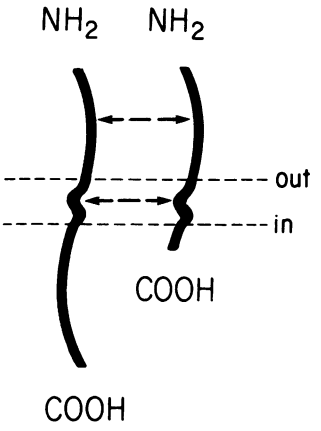

Figure 4. Schematic model of the physical interaction between the EPO-R and the gp55 of the Friend spleen focus-forming virus. The EPO-R (507 amino acids) and the gp55 (409 amino acids) are type I membrane-spanning proteins. Protein–protein interaction may occur either in the membrane-spanning regions of the two proteins or in the extracytoplasmic domains of the two proteins. The interaction stimulates the EPO-R signaling and therefore mimicks EPO binding.

anchor. The interaction between the EPO-R and gp55 could occur at several different sites along the molecules (Fig. 4) and in several possible subcellular compartments. Through direct binding of the EPO-R, gp55 can stimulate the receptor and bypass the normal requirement for EPO, causing a prolonged proliferation of infected erythroid cells.

The gp55 may mimic EPO itself, although there is no amino acid homology shared between these two proteins. Alternatively, gp55 may have sequences derived from (and therefore mimic) the normal cellular second subunit of the EPO-R. The interaction between the EPO-R and gp55 is also observed in MEL cells (58) and probably accounts for the absence of high-affinity binding sites on MEL cells and for the EPO unresponsiveness of these cells (41). Recent evidence suggests that the interaction between the EPO-R and the gp55 occurs within the endoplasmic reticulum (58, 64). Also, the interaction of these two proteins is a novel mechanism of viral transformation leading to growth factor independence. It is possible that analogous retroviruses transform other cell types using, for instance, the IL-2 receptor or the IL-3 receptor, other members of the EPO-R superfamily.

Future directions

The recent cloning of the EPO-R, its membership in a new cytokine receptor family, and its interaction with the gp55 all suggest new directions for erythropoiesis research. The EPO-R, for instance, provides an important stage-specific marker of erythroid differentiation. It is possible that expression of the EPO-R in an early hematopoietic cell (say, BFU-E) actually locks the cell into an erythroid differentiation pattern. Future studies will determine the time during red cell development that the EPO-R is expressed and how this expression changes during differentiation. Hematopoiesis is likely to be accompanied by the sequential expression and loss of the EPO-R and other growth factor receptors.

There are two classes of human diseases that, theoretically, could result from defects in the EPO-R. First, the EPO-R may have a role in the pathogenesis of pure red cell aplasia including congenital aplastic anemia (so-called Diamond-Blackfan anemia) (65, 66). With the availability of the human cDNA and gene for the EPO-R (67) and antisera which recognize the normal human EPO-R polypeptide (67), the possible role of EPO-R in the pathogenesis of these anemias will be testable. This will lead to better classification of anemias and could lead

to more tailored therapies. Although the EPO-R gene has recently been localized to human chromosome 19p (68), no human diseases suggestive of EPO-R pathology map to this region.

Secondly, polycythemia vera (PV) may be caused by an acquired change in the EPO-R. PV is a human myeloproliferative disorder characterized by an expansion of all three major hematopoietic cell types in peripheral blood and bone marrow, with the major clinical feature being an expanded red cell mass in the setting of low serum EPO levels (69). Erythroid colonies (BFU-E and CFU-E) from bone marrow of PV patients may be grown in semisolid medium in the absence of exogenous EPO whereas growth of such progenitors from normal bone marrow is absolutely dependent on the presence of EPO. Interestingly, radiolabeled EPO binding performed with bone marrow from PV patients reveals only a low-affinity receptor (70), a physiological state reminiscent of MEL cells which co-express the Friend SFFV gp55 and the EPO-R. It is quite possible that the molecular mechanism of PV, involving the loss of the high-affinity EPO-R, will be similar to the constitutively activating mechanism in mouse erythroblasts, induced by the physical association of the gp55 and the EPO-R. It is possible that bone marrow cells from PV patients express a viral or host protein which constitutively activates the endogenous EPO-R.

The recent cloning of the EPO-R and the recognition of the new cytokine receptor family will allow a more directed approach to studying EPO-induced signal transduction. Before the cloning of EPO-R, several conflicting studies addressed the question of possible second messengers. The EPO-R has significant amino acid homology (35%) in the cytoplasmic domain with the IL-2R (p75) and the IL-3R, receptors recently shown to transduce signaling through tyrosine phosphorylation. For instance, the IL-2R (p75) subunit activates a tyrosine kinase activity and the p75 subunit is itself tyrosine phosphorylated (71). It is possible that the cytokine receptors are noncovalently associated with a tyrosine protein kinase in a manner similar to that observed with the T cell surface antigen, CD4 (72, 73). The cytoplasmic homology of the EPO-R with these other receptors strongly argues for protein phosphorylation as an early component of transmembrane signaling. Also, because the EPO-R, transfected into IL-3–dependent cells, confers EPO dependence on these cells, a common pathway of signaling is likely to be found for these receptors and for other receptors in the cytokine receptor superfamily.

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