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The molecular biology of erythropoietin

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Abstract Erythropoietin (Epo) controls the proliferation, differentiation and survival of the erythroid progenitors. Epo exerts its effects by binding to a cell surface receptor. The Epo receptor includes a p66 chain, which is dimerized upon Epo activation, and two accessory proteins, which have been defined by cross-linking. Epo binding induces stimulation of the Jak2 tyrosine kinase. Jak2 activation leads to the tyrosine phosphorylation of several proteins, including the Epo receptor itself. Different intracellular pathways are activated: Ras/MAP kinase, phosphatidylinositol 3-kinase and STAT transcription factors. However, the exact mechanisms by which the proliferation and/or differentiation of erythroid cells are regulated after Epo stimulation are not known. Target disruption of both Epo and Epo receptors showed that Epo is not involved in the commitment of the erythroid lineage; it seems to act mainly as a survival factor. Epo is synthesized largely by the kidney and the liver, and sequences required for tissue-specific expression have been localized in the Epo gene. A 3′ enhancer is responsible for hypoxia-inducible Epo gene expression. Hypoxia-induced factor-1 (HIF-1) protein binds to this enhancer. In addition to anaemia of renal failure, the indication for treatment with epoetin has been extended to the anaemia of chronic diseases.

Key words: erythropoietin; erythropoietin receptor; hypoxia; signal transduction

Introduction

Erythropoietin (Epo), a 34 kDa glycoprotein hormone, was the first haematopoietic growth factor to be cloned. The role of Epo is to control red blood cell production through the promotion of survival, proliferation and differentiation of the erythroid progenitors in the bone marrow. Epo is the only haematopoietic growth factor whose production is regulated by hypoxia. Epo was purified successfully by Miyake *et al.* [1] from the urine of aplastic patients. From tryptic fragments of this urinary Epo, Epo DNA probes were synthesized for the isolation and cloning of the human Epo gene [2,3]. The use of recombinant Epo (epoetin) to treat the anaemia of chronic renal failure followed shortly thereafter [4].

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Role of Epo in erythropoiesis

Cultures of haematopoietic progenitors in semi-solid media have shown that the main targets of Epo are the late erythroid progenitors, especially the colony-forming units-erythroid (CFU-E) [5,6]. Epo and Epo receptor gene disruptions in mice confirmed that Epo stimulation was essential for the survival and proliferation of CFU-E [7,8]. Moreover, these experiments showed that Epo stimulation was not necessary for the commitment of the progenitors in the erythroid lineage. Indeed, both burst-forming units-erythroid (BFU-E) and CFU-E were produced to normal levels in mice that lacked either Epo or Epo receptor genes [7,8], thereby demonstrating that Epo is not involved in the determination of the erythroid lineage. Thus, the overall action of Epo appears to be rescue from apoptosis, sustainment of cell proliferation and induction of erythroid-specific proteins at the CFU-E level.

Epo is able to sustain the proliferation of several haematopoietic cell lines, which either express Epo receptors naturally, such as HCD-57 [9] or UT-7 [10,11], or undergo ectopic expression of Epo receptors [12–16]. However, all these cells undergo apoptosis after growth factor deprivation, thus the essential role of Epo in these cells seems to be the inhibition of apoptosis. HCD-57 cells infected with a retroviral vector encoding either Bcl-2 or Bcl-X_L remain viable in the absence of Epo. However, Epo is still required for the proliferation of these cells [17].

Epo receptor

Due to the very low concentrations of circulating Epo and the difficulty of purifying the naturally produced hormone, study of the Epo receptor did not begin until after the Epo gene was cloned and the recombinant

hormone was produced in 1985 [2,3]. Using iodinated Epo, the Epo receptor was first observed in both normal and transformed erythroid cells (reviewed in [18]). These studies showed that the number of Epo receptors found at the cell surface of erythroid cells was very low, ranging from a few hundred to a few thousand Epo receptors per cell. In the erythroid lineage, the Epo receptor appears to be expressed mainly at the CFU-E/proerythroblast stage [19–21]. Epo receptor numbers decrease during the terminal stages of erythroid differentiation, and reticulocytes and erythrocytes are devoid of Epo receptors [20,22,23]. Epo receptors were also detected in megakaryocytic cells [24,25], in murine placenta [26], in endothelial cells [27,28] and in cells with neural characteristics [29].

The Epo receptor (p66) was cloned by an expression strategy from a cDNA library of murine erythroleukaemic cells [30]. Transfection of p66 induces the proliferation of most growth factor-dependent haematopoietic cells after Epo stimulation. Chemical Epo cross-linking to the cell surface of erythroid cells has revealed the association of Epo with two molecules of 85 and 100 kDa, respectively (reviewed in [18]). The identity of these proteins, their relationship with p66 and their role in the mechanism of action of the Epo receptor are unclear. These proteins are associated with p66 but are not recognized by anti-p66 antibodies [31]. Expression of truncated forms of p66 in transfected haematopoietic cells does not modify the apparent molecular mass of p85 and p100 [32,33]. The same observation was made using the TF-1 cell line, which naturally expresses a truncated Epo receptor [34], thereby confirming that these proteins do not derive from the p66 cloned protein. Thus, the identity of these Epo-cross-linked proteins needs to be determined in order to better understand the structure of the Epo receptor.

In common with most haematopoietic growth factor receptors, p66 belongs to the cytokine receptor family. The extracellular domain of these receptors possesses a conserved domain of $\sim$200 amino acids, which derives from the duplication of a 100 amino acid subdomain with a type III fibronectin structure [35]. These receptors exhibit a WSXWS sequence in the second (membrane-proximal) subdomain and two pairs of cysteines in the first subdomain. The two pairs of cysteines are held by disulphide bonds. The WSAWS sequence of the Epo receptor appears to be necessary for the correct folding and/or transport of p66 at the cell surface. Indeed, mutations affecting each of the WS motifs reduce or inhibit p66 cell surface expression [36,37].

Epo appears to activate the Epo receptor by dimerization of the p66 protein. Dimers of the extracellular domain of p66 bound to one Epo molecule have been demonstrated [38]. The affinity of each binding site was determined: the first Epo receptor molecule bound Epo with a fairly high affinity (K_d 1 nM), whereas the second Epo receptor molecule appeared to bind to the complex with a low affinity ($K_d \sim 2\ \mu M$). Direct evidence for the two domain model of Epo function was provided recently by the restoration of biological activity to an inactive Epo mutant following its homodimerization [39].

In addition to Epo binding, the Epo receptor can be activated in several other ways. Bivalent but not monovalent (Fab fragments) anti-p66 antibodies have been reported to activate the Epo receptor [40,41], probably by dimerization of the p66 protein. Small peptides able to mimic the biological effects of Epo have also been isolated. These peptides do not present any sequence homology with the Epo molecule [42]. The crystal structure of the complex of one of these agonist peptides with the extracellular domain of p66 shows that a peptide dimer induces the dimerization of p66 [43]. The covalent dimerization of this peptide recently was shown to increase its potency [44].

An activating mutation in the extracellular domain of p66, changing the Arg129 residue to Cys, constitutively activates the Epo receptor [45] and induces leukaemia in mice [46,47]. The Cys residue is held in a disulphide bond, which is believed to homodimerize the p66 molecule [48]. The mutation of other amino acids (Glu132 or Glu133) to Cys in the same region of p66 also produces constitutively activated Epo receptors [49].

Epo-induced intracellular signalling

Like all the receptors of the haematopoietic receptor family, the Epo receptor does not possess endogenous tyrosine kinase activity. Despite this fact, Epo stimulates the rapid tyrosine phosphorylation of a number of proteins. The first step of intracellular signalling is the activation of the Jak2 tyrosine kinase, which is constitutively associated with the Epo receptor [50] (Figure 1). Jak2 is known to associate with the Epo receptor in a region close to the transmembrane, and deletion of this part of the Epo receptor inhibits Epo-induced cellular proliferation [51].

Among the proteins phosphorylated on tyrosine residues in response to Epo is the Epo receptor itself [13,52–54]. Eight tyrosines located in the cytoplasmic domain of the Epo receptor are probably phosphorylated after Epo stimulation. These phosphorylated tyrosines are in turn docking sites for various intracellular proteins containing Src homology 2 (SH2) domains. After binding, these proteins subsequently can be tyrosine phosphorylated and activated. Therefore, stimulation by Epo leads to localization close to the receptor and the plasma membrane of different activated molecules which participate in downstream signal transduction (Figure 2).

The Ras/MAP kinase pathway is activated by Epo [55,56]. This pathway is involved in cell proliferation in response to Epo [57].

A direct association between phosphatidylinositol 3-kinase (PI 3-kinase) and the Epo receptor has been shown [58–61], which involves the SH2 domains of the p85 subunit of the PI 3-kinase and the last tyrosine

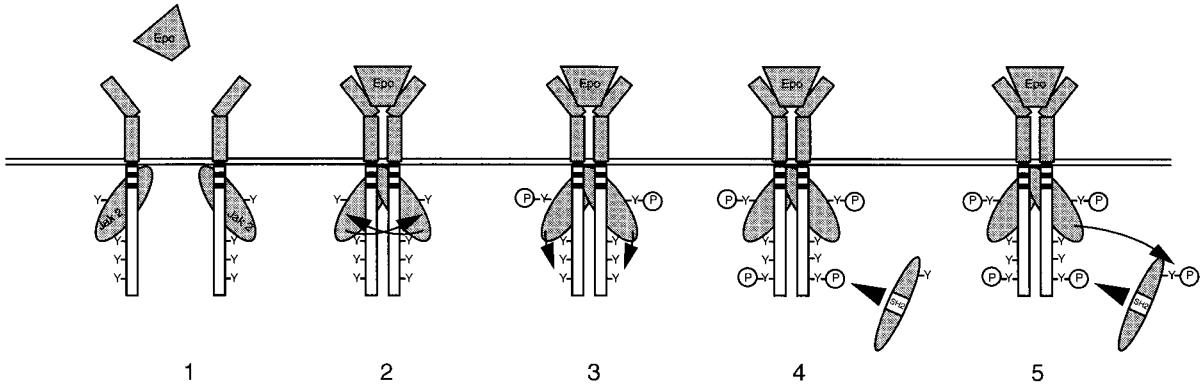

Fig. 1. Epo activation leads to dimerization of the Epo receptor (1); the pre-associated Jak2 kinases are in close contact and activated by transphosphorylation (2). The tyrosine residues of the Epo receptor are then phosphorylated (3, 4), providing docking sites for intracellular signalling proteins with Src homology 2 (SH2) domains (5).

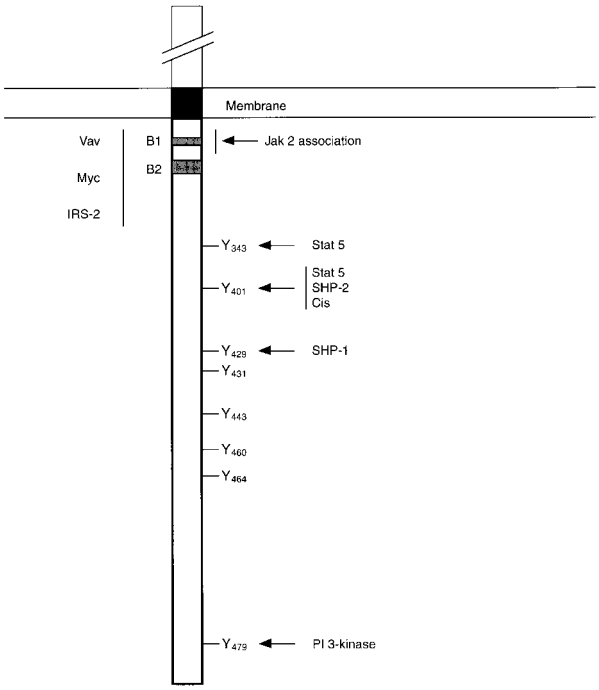

Fig. 2. Schematic representation of the intracellular part of the Epo receptor and the identified binding sites for signalling proteins.

of the Epo receptor [62]. An alternative pathway for the activation of PI 3-kinase has been described recently, which involves the tyrosine phosphorylation of the adaptor protein IRS2 and its subsequent association with PI 3-kinase; this mode of activation therefore does not require the interaction of PI 3-kinase with the Epo receptor tyrosines [63].

The STAT (signal transducer and activator of transcription) pathway plays a major role in cytokine-induced signalling [64]. Epo activates both STAT5A and STAT5B [65–67]. The two first tyrosines of the intracellular domain of the Epo receptor (Tyr343 and Tyr401) are responsible for STAT5 fixation and activation [68–72]. Despite a large number of publications, the precise role of STAT5 in the signalling induced by Epo is the subject of controversy. Whereas a correlation between STAT5 activation and cell proliferation has been described by some groups [68,69,73], others did not obtain such results [71,72]. Furthermore, a correlation between STAT5 activation and Epo-mediated erythroid differentiation was observed in some reports [74,75], while the opposite was shown by others [73].

Two tyrosine phosphatases, SHP-1 and SHP-2, play a role in Epo-induced signalling. SHP-2 is phosphorylated on tyrosine in response to Epo and associates with the second tyrosine residue of the Epo receptor (Tyr401); SHP-2 seems to play a positive role in stimulating cell proliferation [76]. In contrast, SHP-1 plays a negative role in Epo-induced signal transduction; its association with Tyr429 of the Epo receptor leads to the dephosphorylation of Jak2 [77]. Interestingly, De La Chapelle *et al.* [78] described a familial erythrocytosis in which a truncated Epo receptor was found in the polycythaemic members of the family; these truncated receptors were shown to be hypersensitive to Epo probably because they lacked the SHP-1-binding site.

The role of tyrosine phosphorylation of the Epo receptor in Epo-induced signalling is debated. An Epo receptor completely devoid of tyrosine residues is still able to transduce a proliferative signal; some reports have found that these Epo receptors were less sensitive to Epo [33,68,69,79], whereas this decrease in sensitivity has not been mentioned by others [70–72]. Expression of β-globin can be obtained in Ba/F3 cells after transfection of mutant Epo receptors that do not contain any tyrosine residue [33,79]. However, in normal erythroid progenitors, the last tyrosine of the Epo receptor cytoplasmic domain (Tyr479) seems to be required for erythroid colony formation [80,81]. This Tyr479 is sufficient to obtain erythroid differentiation of progenitors from fetal liver [80]; similar properties were reported recently by Longmore *et al.* in an *in vivo* model [82].

Another haematopoietic growth factor receptor belonging to the tyrosine kinase receptor family, the

stem cell factor (SCF) receptor or c-kit, seems to interact with the Epo receptor. It was shown that this receptor associated with the extended box2 region of the Epo receptor and could activate and phosphorylate the Epo receptor, thus enhancing erythroid cell proliferation and differentiation [83]. In addition, this cooperation between c-kit and Epo receptors has been found to be essential for the normal erythroid differentiation of progenitors derived from fetal liver [81]. The exact mechanism of interaction between these two receptors is, however, unclear; they appear to act through distinct intracellular signals [84].

Regulation of Epo production

Epo production is regulated by hypoxia, which leads to an increase in Epo gene transcription [85]; there are no stores of pre-formed Epo. Control of Epo gene expression involves complex interactions between DNA and nuclear proteins. A 50 bp hypoxia-inducible enhancer has been defined approximately 120 bp 3′ to the polyadenylation site, and is responsible for hypoxia-inducible Epo gene expression [86–88]. Mice transgenic for a construct containing the Epo gene and this 3′ enhancer harboured hypoxia-inducible Epo gene expression in the liver.

The 3′ enhancer contains three different segments [89]. A conserved sequence located near the 5′ end of the enhancer is the binding site for a novel transcription factor designated hypoxia-induced factor-1 (HIF-1) [90,91]. The middle segment is less conserved between species, but seems to play a role in the inducibility of both the human and the murine Epo enhancers [92]. The third part corresponds to 3′ DNA sequences, which are binding sites for the hepatocyte nuclear factor 4 (HNF-4) [93]. Furthermore, the C-terminal portion of HIF-1 specifically binds to p300 and overexpression of p300 enhances hypoxic induction [94]. Thus, it is likely that hypoxia induces the formation of a large complex of proteins directly or indirectly bound to the enhancer, which in turn transduce a signal to the Epo promoter, thereby permitting gene transcription [95].

The identification of HIF-1 as a DNA transcriptional complex has been a critical step towards understanding the enhancer function. Affinity purification showed that HIF-1 is composed of two subunits [89,96]. Molecular cloning of HIF-1 by Wang and Semenza [97] showed that the DNA-binding complex was composed of two basic helix–loop–helix proteins called HIF-1α and HIF-1β. HIF-1β previously had been identified as the aryl hydrocarbon nuclear receptor translocator (ARNT), a molecule involved in the xenobiotic response [98]. In contrast, HIF-1α was a new member of this family of proteins. In hypoxic conditions, the levels of the mRNAs encoding either HIF-1α or HIF-1β were not altered, suggesting that the activity of the HIF-1α–ARNT complex is regulated by a post-transcriptional mechanism and a conformational change after recruitment of the ARNT transcription factor [99]. Furthermore, another step of regulation of HIF-1α involves the ubiquitin–proteasome system in its proteolytic destruction in normoxia, while it accumulates rapidly following exposure to hypoxia [100].

The mechanism of regulation by hypoxia was first studied in hepatoma cells, Hep3B or HepG2, which produced Epo. It was shown further that identical responses could be obtained in a large array of non-Epo-producing cells and that the system of gene regulation by oxygen was widespread [101,102]. Many genes have now been identified as targets of HIF-1 function. In addition to Epo, these include vascular endothelial growth factor (VEGF), several glycolytic enzymes, glucose-transporter 1, inducible nitric oxide synthase, haem oxygenase and transferrin [103]. These recent data strengthen the idea that cellular response to hypoxia is an important physiological process and that a similar mechanism for oxygen sensing and signal transduction must be shared by many tissues and cells [104]. However, oxygen-sensing mechanisms are still not completely understood. According to the model of Hep3B cells, a single cell type can apparently sense hypoxia and respond by increasing Epo RNA levels [105]. It was proposed that the oxygen sensor is a haem protein that changes its conformation depending on the binding of oxygen to its haem moiety. The iron atom of haem can be replaced by cobalt, thereby mimicking the hypoxic state [106]. The exact mechanism of activation of transcription factors by hypoxia, however, remains to be determined.

Indications of Epo treatment outside the setting of renal anaemia

Epoetin has been used for the correction of anaemia of renal failure. In some cases, this treatment has been extended to anaemia of chronic diseases. In these disorders, cytokines involved in the inflammatory response inhibit both Epo synthesis and erythroid colony formation *in vitro* [107,108]. It was shown recently that interferon-γ (IFN-γ) down-regulates the SCF receptor and the Epo receptor at the surface of the erythroid progenitors, thus leading to reduction of the survival and growth of these cells and eventually to apoptosis of these progenitors [109]. Further studies showed that IFN-γ induced the concomitant expression of Fas and Fas ligand at the surface of the erythroid progenitors, thereby leading to apoptotic cell death [110]. More work is needed to understand if physiological interactions between the intracellular signals induced by Epo and the Fas system play a role in erythroid cell survival. About half of anaemias in patients with chronic anaemia of cancer can be corrected with epoetin. The appropriate selection of patients and cost–benefit ratios are major problems associated with these clinical applications of the hormone.

Chemical modification of the Epo molecule to improve its stability and specific activity would be

interesting, provided that no antibodies arise against this new molecule.

Lastly, studies are ongoing that aim to provide systemic delivery of the hormone by gene therapy. The tightly controlled expression of the transgene is an absolute necessity before this approach could be envisaged for therapeutic use.

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