Structure of the Erythropoietin Receptor

(43742)

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Abstract. **Despite extensive studies, the structure of the erythropoietin receptor remains little understood. cDNAs encoding the human and murine erythropoietin receptors have been cloned and the structure of these proteins is discussed. Although the proteins encoded by these cDNAs play key roles in erythropoietin binding and in erythropoietin signal transduction, increasing evidence strongly suggests that the erythropoietin receptor is a multimeric complex. The murine erythropoietin receptor has been solubilized under mild conditions and the molecular mass of the native receptor has been shown to be significantly higher than the molecular mass of the cloned chain. Cross-linking experiments have revealed the presence of three proteins covalently bound to erythropoietin by the cross-linking reagents; however, only one of them seems to derive from the cloned chain. Moreover, functional evidence also suggests the presence of other erythropoietin receptor subunits.**

[P.S.E.B.M. 1994, Vol 206]

Erythropoietin (Epo) is a glycoprotein hormone produced primarily by the kidney and is the principal factor promoting viability, proliferation, and differentiation of erythroid progenitor cells (see 1 for a recent review concerning Epo). On sensitive cells, Epo binds to specific cell surface receptors. The study of the Epo receptor structure is hampered by the low number of Epo binding sites present on erythroid cells. Indeed, normal erythroid progenitors exhibit less than 1,000 Epo receptors per cell (2). Some Epo responsive, transformed cell lines with erythroid characteristics present an increased number of Epo binding sites and genetic alterations of the Epo receptor genes (3; Chretien *et al.*, submitted). However, even in these cells, the total number of Epo receptors present on the cell surface does not exceed 10,000 per cell.

Structure of the Cloned Chain of the Epo Receptor

In 1989, a cDNA encoding the murine Epo receptor was cloned from a murine erythroleukemia cell line by an expression strategy (4). The encoded protein is a 507 amino acid Type I membrane spanning protein. The first 24 amino acids form a signal peptide and the remaining 483 amino acids, with a theoretical molecular mass of 52 kDa, are organized in an extracellular hormone binding domain of 223 amino acids, a transmembrane domain of 24 amino acids, and an intracellular domain of 236 amino acids. The extracellular domain of the Epo receptor shares two distinctive features with other members of the cytokine receptor family: a set of four cysteine residues and a five-residue motif close to the transmembrane domain, Trp-Ser-X-Trp-Ser. The cytoplasmic domain is devoid of a consensus sequence for kinase activity. The cytokine receptor family includes the receptors for interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia-inhibiting factor (LIF), growth hormone, prolactin, ciliary neurotrophic factor, as well as c-*mpl*, the cellular homolog of the oncogene transduced by the mouse myeloproliferative leukemia virus.

Transfection of the Epo receptor cDNA into IL-3-dependent hematopoietic cells conferred to these cells the ability to grow in response to Epo. Metabolic labeling of these cells, using [^{35}S]methionine and immunoprecipitation with antibodies directed against the cloned chain, revealed that the Epo receptor protein is synthesized as a 62-kDa unglycosylated form (5).

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0037-9727/94/2063-0200$10.50/0

Thus, the unglycosylated form of the Epo receptor exhibits a molecular mass 10 kDa higher than the theoretical molecular mass calculated from the primary sequence of the cloned chain. Whether this difference is due to an anomalous migration on polyacrylamide gels or to post-translational modifications other than glycosylation remains unknown. The 62-kDa unglycosylated form is successively processed by glycosylation to a 64-kDa high mannose N-linked oligosaccharide form, and lastly, to a 66-kDa complex-type sugar form. Hereon, this protein will be referred to as p66. Ligand blotting experiments identified a 61- to 65-kDa protein from human and murine erythroid cells able to bind Epo (6). Although the identity of this protein was not fully established, it most probably corresponded to p66. In transfected hematopoietic cells, most of the receptors remain inside the cells either in the 64-kDa or in the 66-kDa forms and do not reach the cell surface (5). By analogy to the T cell receptor complex (7), the cell surface expression of p66 may be dictated by the presence of accessory subunits (see below). A highly glycosylated form of 70–78 kDa was also described and tentatively correlated with the biologically active form of the receptor (8).

Characteristics of Epo Binding

Scatchard analysis of Epo binding curves revealed that some cells express both high-affinity (K_d = 100 p*M*) and low affinity (K_d = 600 p*M*) Epo binding sites whereas other cell types only express a single class of binding sites with K_d ranging from 100 to 600 p*M* (see 9 for extensive presentation). It has been suggested that the high-affinity binding sites mediate the biological effects of the hormone (10). However, it now seems likely that there is no strict correlation between the presence of high-affinity binding sites and the ability for cells to respond to Epo. Indeed, Epo-receptor-transfected COS cells which are insensitive to Epo show both high- and low-affinity Epo receptors (4), whereas UT-7 cells (11) or human (12) and rat (2) erythroid progenitors which are Epo responsive, exhibit only a single class of Epo receptors. The biochemical modifications leading to these various affinities of the Epo receptor are unknown. A recent report shows that tunicamycin treatment of cells expressing both high- and low-affinity Epo binding sites, converted the biphasic Scatchard plot to a single phase with high-affinity sites (13). However, the glycosylation state of the cloned chain of the Epo receptor does not appear to be involved in tunicamycin action since mutation of the asparagine in the single N-glycosylation sequence of p66 does not change the affinities for Epo nor the tunicamycin action (13).

The Epo receptor has been reported to be naturally expressed in cells with neural characteristics like PC 12 or SN 6 (14). Although Epo receptor cDNA cloned from PC 12 cells and from rat erythroid cells was identical, the Epo binding sites of PC 12 present an affinity nearly 100-fold lower than that of erythroid cells. These results strongly suggest the presence of a putative accessory protein(s) that may modify the ligand binding affinity. This hypothesis was recently strengthened by a report of Dong and Goldwasser (15) showing that fusion of CHO cells, which have no receptors for Epo with erythroleukemia derived IW 201 cells, which have only low-affinity receptors, resulted in high-affinity binding sites for Epo.

The isolated extracellular part of p66 alone is able to bind Epo (16, 17). Single slope Scatchard plots were reported but the affinities were significantly lower than those described for cellular Epo receptors. Particularly, the dissociation kinetics appear to be much faster (17).

Biochemical Evidence for a Multimeric Structure for the Epo Receptor

To date, the purification of Epo receptors from erythroid cells has not been reported. We have solubilized with Triton X100 nondenatured Epo receptors from erythroid cells isolated from the spleens of mice infected with the anemia strain of the Friend virus (18). Hydrodynamic properties of the solubilized complex have been determined by a combination of gel filtration chromatography and ultracentrifugation through sucrose gradients made in H_2O and D_2O. The Epo-Epo receptor-Triton complex exhibited a Stokes radius of 7.7 nm and a sedimentation coefficient of 11.7S. The detergent contribution was estimated from the apparent density of the complex. From these results, a minimum molecular mass of 330 ± 50 kDa was calculated for the Epo-Epo receptor complex, strongly suggesting a multimeric structure for the Epo receptor.

Many cross-linking experiments have been reported using various erythroid cells and [^{125}I]labeled Epo (see Table I of Ref. 9 for extensive presentation). With the exception of cells infected with the polycythemia strain of the Friend virus (see below), all these experiments revealed that Epo can be cross-linked with the homobifunctional cross-linker dissuccinimidyl suberate (DSS) to two proteins of about 85 (p85) and 100 (p100) kDa molecular masses. In addition, the presence of faint bands corresponding to the association of Epo to a 63–66 protein was also sometimes reported (19–21). When 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDAC) was used instead of DSS, cross-linking experiments clearly revealed the presence of three Epo cross-linked proteins with molecular masses of 66, 85, and 100 kDa (Fig. 1).

The relationship between these proteins is not clearly understood. P85 and p100 are poorly glycosylated and thus cannot derive from p66 by glycoslyation (22). Peptide mapping of p85 and p100 shows that they

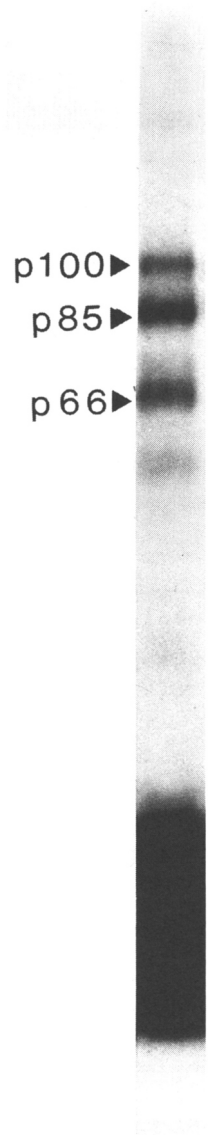

Figure 1. [^{125}I]Epo cross-linking in the cell surface of erythroid cells. Reds 5 Rauscher erythroid cells were labeled with [^{125}I]Epo, cross-linked with EDAC, and solubilized using Triton X100. The solubilized proteins were immunoprecipitated using anti-p66 antibodies and immunoprecipitates were separated by polyacrylamide gel electrophoresis as described elsewhere (21).

are similar or identical in structure, suggesting that they may derive from the same protein (23). Some authors have shown that p85 and p100 can form disulfide bonded complexes of high molecular mass (2, 19, 24). However, this result was not obtained by other groups (25). It has been suggested that p85 and p100 could derive from the cross-linking of more than one protein with Epo (4, 6). However, because cross-linking efficiency is rather low, the probability of two cross-linking with the same Epo molecule is unlikely. Moreover, this hypothesis was studied by using a radioiodinated, cleavable cross-linker: the Denny-Jaffe reagent (DJ). Epo was attached to one end of [^{125}I]DJ and incubated with erythroid cells. The other end of the cross-linker was activated by ultraviolet light to react with the receptor. Then, DJ was cleaved by sodium dithionite, removing the Epo molecule but transferring the iodine label from the Epo molecule to the receptor molecule. Both the 85- and 100-kDa proteins were labeled (26). Thus, p85 and p100 do not derive from artifactual cross-linking of several proteins with Epo.

p66, p85, and p100 were immunoprecipitated by an antiserum directed against p66 from native Triton solubilized receptors. However, only p66 is immunoprecipitated after denaturation of the complex. These data are consistent with the hypothesis that p85 and p100 do not derive from the cloned chain (21). After the transfection of p66 into IL-3-dependent cell lines such as FDC-P1 cells (27) or BaF3 cells (28; Pallu S, Mayeux P, unpublished results), Epo can be cross-linked by DSS with proteins corresponding to p85 and p100 (and also to a protein of 120 kDa in FDC-P1 cells). When these cells are transfected with truncated forms of the p66, the molecular masses of the Epo cross-linked proteins remain unchanged (27; Pallu S, Mayeux P, unpublished results). This result strengthens the hypothesis that p85 and p100 do not derive from the transfected Epo receptor cDNA. Taken together, these data strongly suggest that the Epo receptor present on the membrane of erythroid cells is a multimeric complex composed of a ligand binding component, p66, associated with two proteins, p85 and p100, unable to bind Epo in the absence of p66. The low efficiency of DSS to cross-link Epo to p66 can be explained by the low number of amino groups present on the extracellular region of p66. Indeed, the extracellular region of p66 contains only three lysines, all located close to the NH2 end of the molecule. In contrast, a high number of carboxylic groups are spread along the entire extracellular region and thus the cross-linking of Epo with p66 is easier using carbodiimides.

It has been shown that the Epo receptor can associate with the envelope protein (gp55) of the defective virus of the Friend virus complex (SFFVp). This association leads to the continuous activation of the Epo receptor in infected erythroid cells (29). The interaction between these two molecules appears to involve their transmembrane and extracellular domains (30–32). Although most of the gp55-Epo receptor complexes remain in the endoplasmic reticulum (5), a small part of these complexes reaches the cell surface where they can bind Epo (33, 34). Evidence has been reported suggesting that the complexes expressed at the cell surface are responsible for the mitogenic stimulus (34). Epo can be cross-linked to the cell surface gp55-p66 complexes by DSS. Again, p66 was not cross-linked to Epo by DSS. Indeed, the main Epo-cross-linked protein was the retroviral gp55 (33, 34). The cell

surface form of gp55 is a disulfide bridged homodimer (35) and we have shown that in SFFVp-infected erythroid cells, at least some of the gp55 bound p66 was in fact associated to a disulfide bridged dimer of gp55. It is tempting to speculate that each gp55 molecule of the dimer is associated with one p66 molecule. As it has been suggested that the activation of the Epo receptor could involve the dimerization of the receptor (36, 37), this mechanism could account for the Epo receptor activation by gp55. Cross-linking experiments also showed that the binding of gp55 to p66 apparently partially displaces the 85- and 100-kDa proteins from the Epo receptor complex (33, 34).

The structure of the Epo receptor was also studied after transfection of p66 in COS cells. In contrast to erythroid cells or to hematopoietic cells transfected with p66, p66-transfected COS cells exhibit a high number of Epo binding sites (4). In these transfected cells, Epo bound to the cell surface can be cross-linked by DSS with two proteins. One of these proteins exhibits a molecular mass of 65 kDa and most probably corresponds to p66. There is some discrepancy concerning the molecular mass of the other Epo cross-linked protein which was reported to be nearly 105 kDa (4, 38) or 88 kDa (28). It was initially proposed that the 88- to 105-kDa protein corresponded to one of the proteins cross-linked to Epo in erythroid cells. However, this assumption was never experimentally tested. This protein is directly recognized by anti-p66 antibodies, and it has been suggested that it could correspond to p66 bound to a 35-kDa component (38). Obviously, the determination of the nature of this cross-linked complex will necessitate further studies, especially a more accurate comparison of its properties with those of the Epo cross-linked proteins from erythroid cells.

Functional Evidence for a Multimeric Structure

Although the formation of heteromultimeric complexes is needed to constitute biologically active receptors for most of the receptors of the cytokine receptor superfamily, it has been suggested that the biologically active forms of the G-CSF receptor, the growth hormone receptor, and the Epo receptor are homodimers (36). Indeed, it has been shown that a mutation transforming an arginine residue to a cysteine residue in the extracellular part of the Epo receptor (Position 139) both constitutively activates the Epo receptor and promotes its covalent homodimerization by disulfide bonding (37). However, other results strongly suggest that the presence of components distinct from p66 are needed to constitute the functional receptor. Chiba *et al.* (39) have shown that when BaF3 cells are transfected either with the IL-2 or the Epo receptor and stimulated by their respective ligands, each ligand induces the phosphorylation of an overlapping but distinct set of proteins. When they transfected these cells with a chimeric receptor composed by the extracellular domain of the Epo receptor and the cytoplasmic domain of the IL-2 receptor, the tyrosine phosphorylation pattern observed after Epo stimulation corresponded to that induced by the Epo receptor. In contrast, a chimeric receptor composed by the extracellular domain of the IL-2 receptor and the cytoplasmic domain of the Epo receptor transduced an IL-2-type phosphorylation signal. Thus, these results indicate that the extracellular parts of these receptors specify, at least in part, the pattern of protein phosphorylation induced by these receptors.

In contrast to the IL-3-dependent cell line, BaF3, the IL-2-dependent T cell line, CTLL-2, does not proliferate under Epo stimulation after transfection with p66 (40). Sakamaki *et al.* (41) transfected CTLL-2 and BaF3 cells with a chimeric receptor composed by the extracellular domain of p66 linked to the intracellular domain of the IL-3 receptor β chain. This chimeric receptor was able to transduce a proliferative signal in BaF3 cells but not in CTLL-2 cells, although the β chain of IL-3 receptor is functional in CTLL-2 cells. These studies provide evidence that p66 associates by its extracellular domain, or via the bound Epo molecule, with an additional component involved in signal transduction which is lacking in some cells like CTLL-2 cells.

Conclusion

Obviously, the structure of the biologically active Epo receptor complex is not fully understood. Although p66, the cloned chain, plays a pivotal role in both Epo binding and Epo signal transduction, the presence of additional component(s) involved in both Epo binding and signal transduction is needed to explain many results. The two proteins evidenced in cross-linking experiments, p85 and p100, are good candidates for these functions. However, it is now necessary to characterize these proteins using more direct methods than chemical cross-linking to approach their possible role in the mode of action of Epo.

The authors thank Dr. Françoise Porteu for the critical reading of this manuscript. The authors are supported by research grants from the "Association pour la Recherche sur le Cancer" (ARC Contract n° 6327).

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