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Structural and functional characterisation of recombinant human erythropoietin analogues

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Deletion mutants of a synthetic human erythropoietin-cDNA were transiently expressed in COS-7 cells and products analyzed using Western blots and a cell proliferation assay. Only two mutants with deletions affecting the N-terminal portion and the amino acid sequence 115–121 displayed biological activity. The exchange of hydrophilic, charged amino acids by alanine in two potential α-helical regions, the internal amino acid sequence 102–106 and the C-terminal sequence 154–159, causes a 2–11-fold loss of activity. The results suggest that both regions are involved in either maintaining the active structure of the hormone or interacting with the receptor.

Erythropoietin; Mutagenesis; Transient expression; Structure–function relationship

1. INTRODUCTION

The proliferation and differentiation of erythroid progenitor cells are regulated by the glycoprotein hormone erythropoietin [1]. Although the recombinant protein is available and in clinical use for some years many questions concerning the molecular mechanism of EPO's action remain to be answered. Further understanding of its function may be gained from the analysis of the interaction with the EPO-receptor and the signal transduction pathways induced.

A number of studies have shown that the glycosylation, which accounts for 40% of the molecular mass, is responsible for the in vivo stability, cellular processing and secretion [2,3,4,5]. The protein portion of the molecule is essential for interaction with the receptor. Information available on the location of the receptor binding site has been generated by strategies involving anti-peptide antibodies [6,7,8], mutational analysis [9,10,11] and by comparing the sequences of EPO genes from different species [12]. Extensive studies with anti-peptide antibodies show that only those which are derived from peptides covering an internal region (amino acids 99–119; 111–129) and the C-terminal portion (amino acids 152–166) are able to neutralize the biological activity of EPO [7,8]. Deletion of amino acids in regions predicted to have a α-helical structure result in the expression of proteins which are not exported by the cells, whereas mutations within the interhelical loops do not affect the export and biological activity of the products in most cases [9,10,11]. A study on the region between amino acids 99–129 provides evidence that changes to residues 99–110 lead to an inactivation of EPO [11].

We have further characterized the role of regions predicted to be functionally important and shown that deletions, even single amino acid substitutions in the C-terminus and residues 102–106, lead to a loss or significant reduction of the biological activity suggesting that these regions are directly involved in the binding of EPO to its receptor. Moreover, our results exclude an N-terminal region and the amino acids 115–121 as functionally active portions of the molecule.

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Abbreviations: Epo, erythropoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor.

2. MATERIAL AND METHODS

2.1. *EPO gene synthesis*

A synthetic human EPO gene was generated by a chemoenzymatic strategy.

The amino acid sequence was transferred into a DNA-sequence by a computer program creating as many restriction sites as possible without changing the amino acid composition. The coding region was assembled by the sequential ligation of eight blocks consisting of a pair of oligonucleotides each. The oligonucleotides were designed to have complementary regions at their 3′ termini and compatible restriction sites to join the blocks. After annealing the oligonucleotide pairs they were filled in with Klenow-polymerase to give full-length double stranded products. Individual blocks were cloned, sequenced and ligated using standard procedures [13].

2.2. *Deletion mutagenesis and site-directed mutagenesis*

The gene synthesis strategy used provided enough possibilities for deletions in the entire coding region. The deletions were introduced using restriction enzymes. The products were subcloned into the plasmid puc19. Site-directed mutagenesis was carried out with a MutaGene M13 in vitro Mutagenesis Kit (Biorad, Richmond, CA) according to a method published by Kunkel [14]. The presence of all mutations was confirmed by sequencing all constructs with the dideoxy

chain termination method [15] and a sequencing kit supplied by USB (Cleveland, OH).

2.3. *Expression vector constructs*

The human EPO mutants were subcloned into the *Eco*RI restriction site of the plasmid pSVSPORT1 (Gibco, BRL) containing the SV40 origin of replication, early transcriptional promoter, small t-intron and polyadenylation signals (Fig. 1). The orientation of the inserts was confirmed by restriction-mapping. Plasmid DNA was prepared by CsCl-gradient centrifugation followed by two extractions with water-saturated isoamylalcohol.

2.4. *Transfection of EPO and its analogues*

The DNA constructs were transfected into COS-7 cells. COS-7 cells were grown to 80% confluence in Dulbecco's Modified Eagle's medium (DMEM) containing 20 mM HEPES and supplemented with 10% fetal calf serum (FCS). Usually, 20 μg CsCl-purified non-linearized plasmid-DNA and 20 μg sonicated salmon sperm DNA were combined with 4×10^6 cells in 800 μl medium supplemented with 10% FCS. The electroporation was performed with a BioRadGenePulser [16]. A capacitance of 960 μF and a voltage of 250 V were found to produce the highest transfection efficiency. Transfections with the vector pSVSPORT1 were included as controls. The single cell suspension was plated in growth medium immediately post transfection. After 24 h of growth as adherent monolayers the medium was replaced by FCS-free medium and the cells incubated for another 24 h. The conditioned medium was harvested and used for the EPO assays.

2.5. *Detection of mutant EPO proteins*

EPO-protein was detected by Western blots using a monoclonal antibody directed against the NH_2-terminal portion of the amino acid sequence supplied by Medac (Hamburg). EPO-containing COS-cell supernatants were size-fractioned by SDS-PAGE using the buffer system described by Laemmli [17] and 10% gels. For Western blot analysis, proteins were transferred to a 0.2 mm membrane (Hybond-ECL, Amersham) by semi-dry blotting. Non-specific binding sites on the filter were blocked by incubation in PBS/0.1% Tween (PBS-T). Blots were incubated with the anti-EPO-antibody (10 μg) in PBS-T containing 1% BSA for 2 h. After washing with PBS-T and incubation with a horseradish peroxidase labelled anti-mouse Ig antibody (Amersham, 1:5000 in PBS-T/1% BSA) for one hour, the blots were washed with PBS and developed with the ECL-system purchased from Amersham. The bands were scanned by an imaging densitometer (GS-670, Biorad). Several exposures were taken to ensure that the signals were in the linear range of film response. The quantification of EPO-protein was performed by the comparative analysis with pure recombinant EPO.

2.6. *Stimulation of hematopoietic cell proliferation*

The biological activity of EPO mutant proteins was measured by an in vitro bioassay using the EPO-sensitive cell line TF-1. This human erythroleukemic cell line was established by Kitamura et al. [18] and kindly supplied by W. Ostertag, Heinrich-Pette-Institut, Hamburg. The cells were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% FCS and 7.5 ng/ml GM-CSF. Exponentially growing cells were washed free of growth factor, maintained 24 h without GM-CSF and exposed to dilutions of the conditioned COS-cell supernatants for an additional 48 h. The proliferation of the cells was measured with the MTT reduction assay essentially as described by Mosmann [19]. The activity of the mutant proteins was determined after Western blot quantification using recombinant human EPO with known specific activity as a standard.

3. RESULTS

A synthetic EPO-cDNA was constructed by a chemo-enzymatical approach. The strategy involved the introduction of additional restriction sites, which were used to generate a series of deletion mutants. Fig. 2 depicts the structure of the mature human EPO-molecule and shows the location of the deletions and the amino acid substitutions as well as the predicted α-helices in its secondary structure.

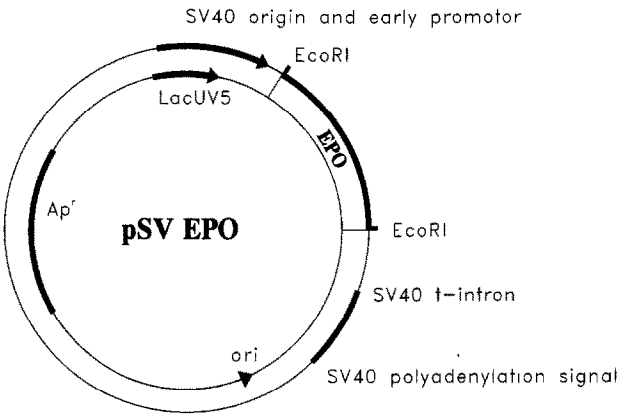

Fig. 1. Expression vector pSVEPO.

The EPO-analogues were designed with regard to earlier reports of mutational analysis [10,11], experiments with anti-peptide antibodies [6,7,8,24] and evolutionary considerations [12].

The amino acids deleted or changed are shown in Table I. Besides the deletion in EPO^d all mutations are located within one of the four predicted α-helices. These α-helices are thought to be critical for the tertiary structure of growth factors and hence may represent the receptor-binding portion of several hematopoietic growth factors, hormones and cytokines [20]. The deletion of the EPO^d mutant is located in the loop joining α-helices C and D. Peptides covering this region were shown to induce antibodies which are able to neutralize EPO's activity [7].

After subcloning the constructs into the expression vector shown in Fig. 1 the cDNA's were transiently expressed in COS-7 cells. By analyzing the supernatants

Table I

Erythropoietin analogues created by deletion and site-directed mutagenesis

Construct	Amino acids deleted or changed	α-Helix position*
EPO^{WT}	–	–
EPO^a	Gln^{13}-Arg^{14}-Tyr^{15}-Leu^{16}-Leu^{17}	A
EPO^b	Gln^{65}-Gly^{66}-Leu^{67}-Ala^{68}-Leu^{69}	B
EPO^c	Leu^{102}-Arg^{103}-Ser^{104}-Leu^{105}-Thr^{106}	C
EPO^d	Gln^{115}-Lys^{116}-Glu^{117}-Ala^{118}-Ile^{119}-Ser^{120}-Pro^{121}	–
EPO^e	Lys^{154}-Leu^{155}-Tyr^{156}-Thr^{157}-Gly^{158}-Gly^{159}	D
EPO^{103}	Arg^{103} → Ala^{103}	C
EPO^{106}	Thr^{106} → Ala^{106}	C
EPO^{154}	Lys^{154} → Ala^{154}	D
EPO^{159}	Glu^{159} → Ala^{159}	D

*Predicted by Bazan [20], see Fig. 2.

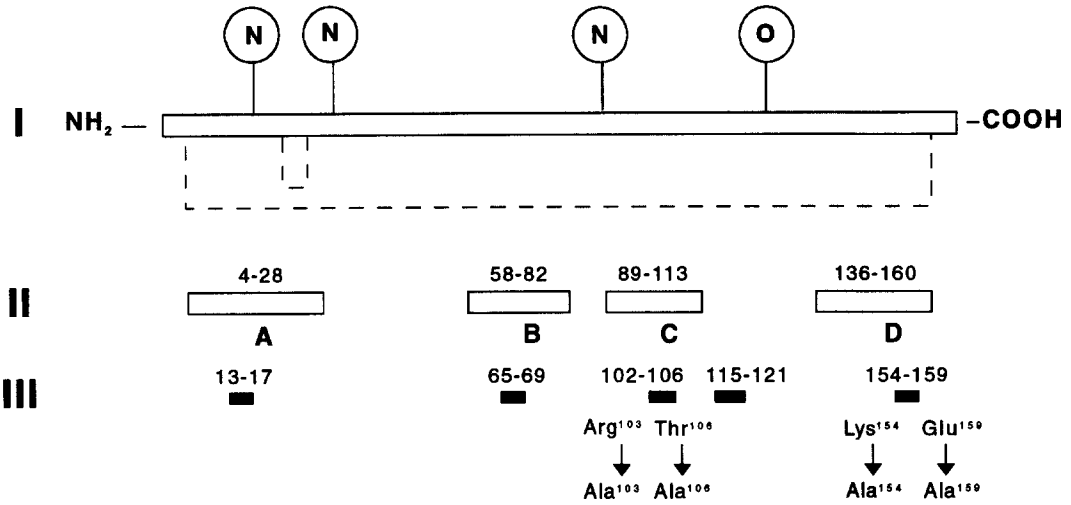

Fig. 2. Structural features of the human EPO-molecule and location of the investigated deletions/amino acid substitutions. (I) Primary structure of the mature human EPO-molecule comprising 165 amino acids; N- and O-linked glycosylation sites are shown by circles; the dashed lines represent the disulphide bonds. (II) Rectangles show the location of the predicted α-helices. (III) Location of deletions and amino acid substitutions that were introduced into wild-type-EPO (WT-EPO).

in Western blots we could show that all the recombinant proteins except EPO^c were synthesized by the COS cells (Fig. 3). In the case of EPO^c (deletion of amino acids 102–106), protein was not detectable in lysed cells (results not shown) as well as supernatants. In comparison, Chern et al. [11] report the inability of COS cells to synthesize detectable amounts of EPO after transfection with mutants affecting the amino acids 99–108. The quantification of the Western blot signals with the help of a standard and densitometric scanning revealed secretion levels in the range of 15–24 ng/ml. The molecular weight of the proteins was in the expected range with a very similar molecular weight heterogeneity, indicating a normal post-translational modification of the products. The supernatants were assayed for their ability to stimulate growth of the EPO-responsive cell line TF-1, as well as their content of immunoreactive protein. The results presented in Table II, indicate that the wild-type cDNA was efficiently expressed and secreted. Only two of five EPO-analogues with amino acid deletions, EPO^a (N-terminal amino acids 13–17) and EPO^d (internal amino acids 115–121), were found to be active. The deletions of C-terminal amino acids 154–159

Fig. 3. Western blot analysis of recombinant human EPO-analogues in COS-7-cell supernatants. Recombinant EPO and the analogues were detected by Western blotting with an anti-EPO-antibody and display the normal size of fully glycosylated EPO. All COS-cell supernatants expressed the transfected EPO gene except EPO^c. Lane 1 = EPO^{wt}; lane 2 = EPO^{103}; lane 3 = EPO^{106}; lane 4 = EPO^{154}; lane 5 = EPO^{159}; lane 6 = EPO^a; lane 7 = EPO^b; lane 8 = EPO^c; lane 9 = EPO^d; lane 10 = EPO^e.

(EPO^e) and two internal sequences (amino acids 65–69/EPO^b; amino acids 102–106/EPO^c) are obviously deleterious for the expression of an active protein. However, the amino acid substitutions affecting the same α-helical regions as the deletions in EPO^c and EPO^e, resulted in detectable, although significantly reduced, activity. The exchange of the hydrophilic amino acids residues Arg^{103}, Thr^{106}, Lys^{154} and Glu^{159} with the neutral amino acid alanine causes a 2–11-fold loss of relative specific activity.

4. DISCUSSION

The aim of this study was to characterize regions of the EPO-molecule which are thought to participate in the interaction with the EPO-receptor and to identify critical amino acid residues involved. In initial experiments internal deletions of 5–7 amino acids were introduced into the wild-type EPO to reveal functionally important domains. Four of the five deletion mutants were effectively expressed as shown by Western blot analysis, but only two of them displayed biological activity.

A mutant with a deletion located in the N-terminal part (EPO^a) had nearly the same biological activity as the wild-type EPO, confirming the observation that antibodies specific for this region do not neutralize the activity of the hormone [6,21]. Another mutant (EPO^d lacking amino acids 115–121) with the deletion in the region which is predicted to join α-helices C and D [20] displayed slightly decreased activity. Similar results were shown by Boissel et al. [9] analyzing EPO-mutants with a deletion of the amino acids 111–119 or an insertion of a myc epitope after Lys^{116}, and by Chern et al. [11] using sequential alteration of the secondary structure defined by the amino acids 120–129.

In contrast, any deletions in this study and reported by others [9,10,11] affecting the α-helical stretches close to the C-terminus (helices C, D) were deleterious for hormonal activity, probably by causing misfolding of the products. The model for the tertiary structure of EPO and several other growth factors and hormones presented by Bazan [20] would suggest that the helical structure near the C-terminus comprises the receptor binding structure. Studies using antipeptide antibodies to hydrophilic regions show that antibodies to the C-terminal amino acids [8], as well as to different peptides covering the amino acids 99–129 [7], are able to neutralize the bioactivity of EPO. In addition, EPO molecules tagged with a hemagglutinin influenza virus epitope or a consensus sequence for phosphorylation at the C-terminus were inactive [22]. From all data available it is evident that the internal and C-terminal regions are necessary to maintain a biologically active structure and are possibly involved in receptor binding.

Deletions of amino acids, as well as the binding of antibodies can lead to gross structural alterations and consequently the inactivation of proteins. Therefore, single amino acid changes were introduced into the molecule in this study to determine if the exchange of residues which are known to be typically involved in receptor interactions, affected the bioactivity. The residues selected for mutagenesis are highly conserved in different EPO-species [12]. Amino acids with hydrophilic, charged side chains were replaced by the neutral amino acid alanine to minimize alterations in the backbone conformation of the recombinant EPO. The lysine residue at position 154 is predicted to be exposed on the surface of helix D which displays conserved sequence patterns in a number of cytokines [20,22]. In each case a loss of specific activity was observed, with the greatest reduction in activity detected in the mutants affecting the region close to the C-terminus.

The data presented here, therefore, indicate the important role of the amino acid sequences 102–106 and 154–159 and define single residues within the helices C and D, which may be involved in the receptor interaction of EPO.

Table II

Biological activity of Erythropoietin in COS-7-cell supernatants

Construct	Biological activity (U/ml)*	Relative specific activity (%)**
EPO^{WT}	3.7 ± 0.4	100
EPO^{a}	3.4 ± 0.5	109
EPO^{b}	n.d.	–
EPO^{c}	n.d.	–
EPO^{d}	2.5 ± 0.4	88
EPO^{e}	n.d.	–
EPO^{103}	1.2 ± 0.3	47
EPO^{106}	1.6 ± 0.3	41
EPO^{154}	0.4 ± 0.1	9
EPO^{159}	0.8 ± 0.2	26

*Determined by a cell proliferation assay (see Section 2) data expressed as means ± S.D. (n = 3)

**Calculated by comparing the quotient of biological activity/immunoreactive protein content for each EPO analogue to the wild type EPO.

n.d. = not detectable.

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