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None of the Four Tyrosine Residues Is Essential for the Biological Activity of Erythropoietin

Homo Son[1], Jin Hyung Lee[2] and Taeowan Chung[2]

[1]School of Pharmacy and [2]Department of Biochemistry, Yeungnam University, Kyeongsan 712-749, Korea

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Erythropoietin (EPO), a glycoprotein hormone, regulates the proliferation and differentiation of erythroid progenitor cells. Many attempts have been made to identify the functionally important amino acids of the hormone. One of those early studies has found that heavy radioiodination of EPO caused the loss of its biological activity, suggesting some important role of one of the four tyrosine residues (Goldwasser, 1981). Thus, in this study, we have generated and tested four Tyr-->Phe substitution mutants to clarify the possible role of the tyrosine residue(s) in the hormone's biological activity. When the mutant and wild type EPO cDNAs were transfected into COS-7 cells and the biological activities of the muteins were assayed using the primary murine erythroid spleen cells, no mutation tested was found to affect the biological activity of the hormone. Thus we conclude that, contrary to the previous observation, none of the four tyrosine residues in erythropoietin is critically involved in the binding of the hormone to its receptor.

Key words : Erythropoietin, Tyrosine residues, Site-directed mutagenesis

INTRODUCTION

Erythropoietin (Epo) is a 31-kDa glycoprotein hormone which regulates the viability, growth, and differentiation of the erythroid progenitor cells (Spivak, 1986). This hormone is produced in the kidney of adults and the serum level of the hormone can be greatly elevated in response to anemia or hypoxia. The protein was purified to homogeniety from the urine of anemic patients and was found to contain carbohydrates as much as 40% of its molecular weight (ca. 31 kDa). The genomic and cDNA clones for mouse and human erythropoietin have been isolated and characterized (Jacobs *et al.*, 1985; Lin *et al.*, 1985; McDonald *et al.*, 1986).

Several approaches have been employed to elucidate the structure-function relationships of the hormone protein. For example, comparison of the amino acid sequences of erythropoietin from various species has demonstrated some highly conserved regions which could reflect functionally important domains of the molecule (McDonald *et al.*, 1986; Lai *et al.*, 1986). The role of oligosaccharide chains in the biological activity of the hormone is still controversial (Dordal *et al.*, 1985; Lai *et al.*, 1986; Sasaki *et al.*, 1987; Wojchowski *et al.*, 1987; Dube *et al.*, 1988; Takeuchi *et al.*, 1988, 1989; Tsuda *et al.*, 1988, 1990; Sytkowski *et al.*, 1991). Antipeptide antibodies have also been employed to find the regions which neutralize the hormone's activity (Sytkowski and Donahue, 1987). Recently, two groups have independently reported, through the site-directed mutagenesis studies, that the arginine 103 is critical in the biological activity of the hormone (Grodberg *et al.*, 1993; Wen *et al.*, 1994). Since one of these early studies has found that radioiodination of the hormone protein resulted in the progressive loss of the hormone's activity, suggesting some functional role of tyrosine residues (Goldwasser, 1981), we have investigated the role of tyrosine residues by substituting them, one by one, with phenylalanine and then determining the biological activity of the muteins. The results presented here, however, show that none of the four tyrosine residues is essential for the biological activity of erythropoietin.

Correspondence to: Taeowan Chung, Department of Biochemistry, College of Natural Sciences, Yeungnam University, Kyeongsan 712-749, Korea.

MATERIALS AND METHODS

Cell culture and transfection

COS-7 cells were kindly provided from Dr. Y.C. Sung of POSTECH and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO). Transient expression of cDNAs

was performed using a DEAE-Dextran protocol modified by 0.1 mM chloroquine treatment (Sussman and Milman, 1984; Ausubel *et al.*, 1989). One day before the transfection, the cells were plated at 1×10^7 cells/100-mm tissue culture dish. 10 μg DNA was used in each transfection. Control experiments for these transfection assays included supernatants from non-transfected cells. Medium was collected 72 hr after transfection and assayed for biological activity and protein amount of erythropoietin.

Measurement of secreted EPOs and their biological activity

Part of the media taken after transient expression was used for quantitative measurement of the hormone by radioimmunoassay using the procedure provided by the manufacturer of the EPO-Track™ RIA Kit (Incstar Corp.). The biological activity of the hormone was determined in vitro by measuring the erythropoietin-induced incorporation of [^{3}H]-thymidine into splenocytes derived from mice treated with phenylhydrazine. The basic protocol was essentially the same as that described by Krystal (1983).

Oligonucleotide-directed mutagenesis

A 1.3 kb EcoRI fragment from pSP70-Epo (a gift from Dr. Arther J. Sytkowski, Harvard Medical School) which contained full length human EPO cDNA, was subcloned into M13mp18 vector using standard procedures (Sambrook *et al.*, 1989). Mutagenesis was performed using the method described by Kunkel (1985), with the following details. The recombinant M13 phage harboring erythropoietin cDNA inserts was used to infect RZ1032, a *dut, ung*$^-$ double mutant strain. After overnight growth, the culture was spun down and the uracil-containing single strand DNA was prepared from the supernatant using polyethylene glycol. The mutagenic oligonucleotide was then hybridized and extended with T4 DNA polymerase. After ligation, the mixture was used to transfect a wild type strain JM101. Plasmid DNA was isolated from the resulting plaques by the alkaline lysis method. The identification of right clones were by restriction enzyme mapping, since the mutagenic oligonucleotides used were designed to lose or generate particuar restriction enzyme sites upon successful mutagenesis. Specifically, the mutants of Tyr 15, Tyr145 and Tyr156 were identified by observing the loss of *Kpn*I, *Acc*I and *Rsa*I sites, respectively, and the Tyr49-->Phe substitution mutant was identified by appearance of a novel *Nru*I site. The mutant EPO inserts were then moved from M13 to the pSV2 vector by replacing the *BstX*I/*Sac*I fragment of wild type EPO insert in pSV2 with the mutant derivatives of the corresponding restriction fragment in M13.

RESULTS AND DISCUSSION

Site-directed mutagenesis and subcloning into an animal expression vector

Since an early study has demonstrated that radioiodination of erythropoietin resulted in the progressive loss of its biological activity (Goldwasser, 1981), it has been generally accepted that one of the tyrosine residues might play a critical role in the hormone's biological activity. Thus, the aim of this study was to clarify the role of the tyrosine residues in the hormonal activity of erythropoietin. We have thus generated four independent Tyr->Phe substitution mutants by site-directed mutagenesis and compared the hormonal activity of these mutant EPOs with that of wild type (Fig. 1). The human EPO cDNA insert used as template for the mutagenesis was derived from pSP70-EPO, where the full length human EPO cDNA sequence including some 5'- and 3' untranslated region, is on the 1.3 kb EcoRI fragment. Mutagenesis was performed on the uracil-containing single-strand template using mutagenic oligonucleotides shown in Table 1. To make the screening procedure easier, all the mutagenic oligonucleotides used were designed to destroy or generate particular restriction enzyme sites by choosing an approapriate codon for phenylalanine out of the two degenerate codons. Specifically, the sequences of the mutagenic oligonucleotides for substitutions of Tyr15, Tyr145 and Tyr156 were designed to lose the *Kpn*I, *Acc*I and *Rsa*I sites, respectively, all of which appear on the wild type sequence. The oligo for Tyr 49-->Phe substitution was 26mer and designed to generate a novel *Nru*I site upon mutagenesis. In this case, the generation of *Nru*I site was possible only with the

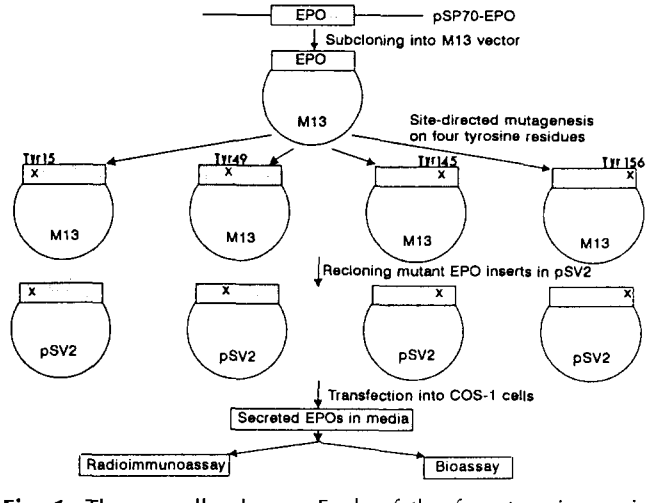

Fig. 1. The overall scheme. Each of the four tyrosine residues was substituted, one at a time, with phenylalanine. The resulting muteins were transiently expressed and assayed for their specific biological activities.

Table 1. Nucleotide sequences of the mutagenic oligonucleotides used

Target Codon	Nucleotide Sequence[a,b]	Restriction Enzyme Site, Gained (+) or Lost (-)
Tyr15	ctccaagaggAacctctccag	(-) *KpnI*
Tyr49	atcctcttccTCgcGAagaaattaac	(+) *NruI*
Tyr145	gaaattggagAagactcggaa	(-) *AccI*
Tyr156	ctcccctgtgAacagcttcag	(-) *RsaI*

[a] The sequence of each restriction enzyme site gained or lost is underlined.
[b] The nucleotides to be changed by mutagenesis are represented in capital letters.

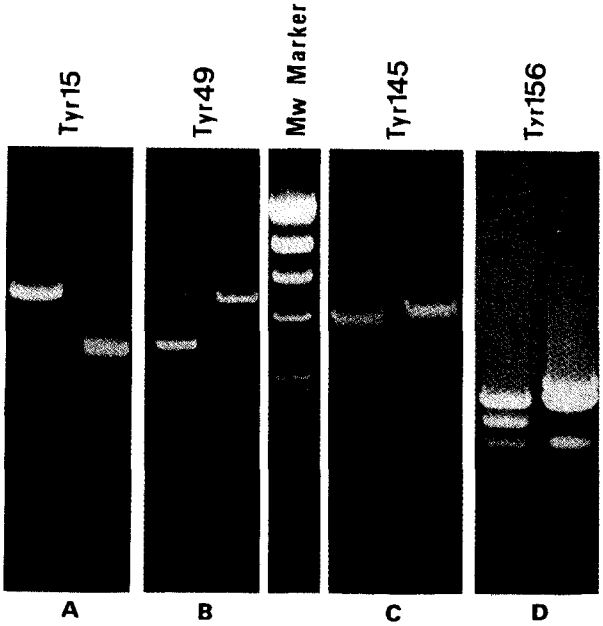

Fig. 2. Analysis of the restriction digestion patterns of four Tyr-->Phe mutant cDNAs in pSV2. After mutagenesis was confirmed by restriction enzyme mapping, the mutant EPO inserts on M13 were moved to pSV2 vector and digested the positive clones with (A) *KpnI*, (B) *NruI*, (C) *AccI* and (D) *RsaI*. In each panel, the left lane is for wild type and the right lane is for mutant.

co-substitution of the Trp51 with Arg. We initially intended to go through another cycle of mutagenesis to restore this unwanted mutation if the double substitution mutant proved to knock out the hormone's biological activity. However, as it turned out otherwise, we didn't put any further effort to correct the adjacent mutation. After positive clones were identified by observing the gain or loss of the particular restriction enzyme sites mentioned above, the mutant EPO inserts were then moved from M13 to an animal cell expression vector, pSV2, by replacing the *BstXI*/*SacI* fragment of wild type EPO insert in pSV2 with the corresponding mutant *BstXI*/*SacI* fragments from M13 vector. Positive clones were again identified by restriction enzyme mapping. As shown in Fig. 2, each mutant derivative of the four tyrosine residues in

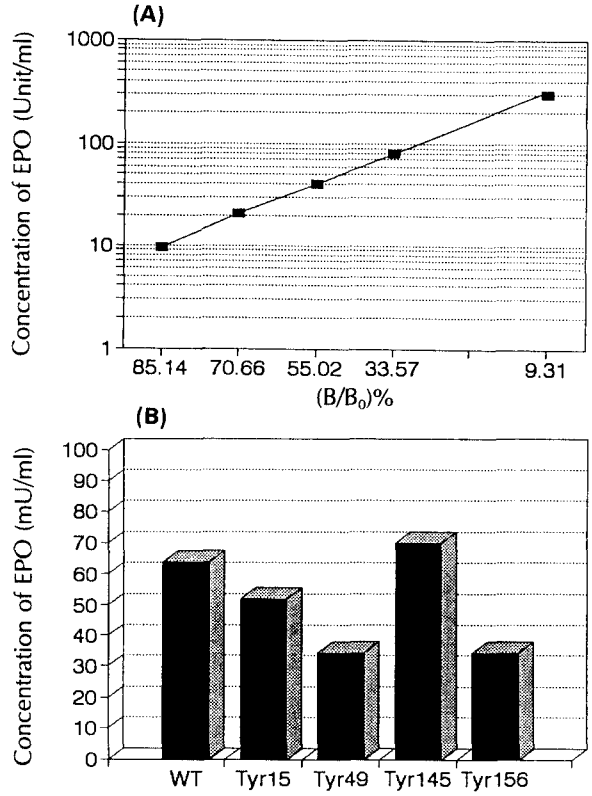

Fig. 3. (A) Standard curve for EPO radioimmunoassay. B/B_0 (%)=(cpm of standard-cpm of nonspecific binding)×100 / (cpm of zero standard-cpm of nonspecific binding) (B) Radioimmunoassay for secreted EPOs from pSV2-EPO^{wt} (WT) and pSV2-EPO^{mt} cDNAs. The wild type and mutant EPO cDNAs on pSV2 were transfected into COS 7 cells and transiently expressed. The amount of secreted hormone proteins were determined by radioimmunoassay.

pSV2 gave distinct restriction digestion pattern compared with that of the wild type. The sizes of the restriction fragments from mutants well agreed with the facts that the mutations occurred at the correct places.

Expression and biological activities of the mutant hormones

pSV2-EPO^{wt} (pSV2 containing the wild type of EPO cDNA) and the four different tyrosine mutants, pSV2-EPO^{mt}, were then transfected into COS-7 cells and were transiently expressed. When the appropriately diluted media containing secreted EPOs, with the concentration adjusted to fall in the range of the standard curve (Fig. 3A), were subjected to radioimmunoassay, all the mutant hormones were found to be normally expressed and secreted (Fig. 3B). A maximum of two-fold difference in the secreted amount of EPOs was observed between mutants and it could come from the difference in either the transfection efficiency or secretion. Since the concentration difference of EPOs between samples was not so great and since it won't put any obstacles in the next bioassay step, we didn't

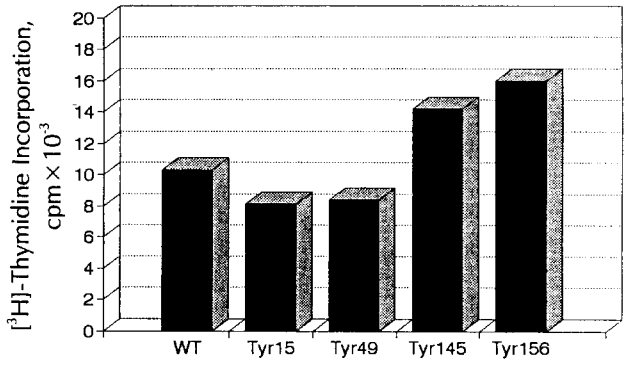

Fig. 4. Specific bioactivities of the mutant hormones. Using fixed amount of hormones, the biological activities were determined by measuring the [^{3}H]-thymidine uptake rate using mouse spleen cells pretreated with phenylhydrazine. Data are representative of three independant measurement.

try to get answer about which is the case. Using the remaining part of the sample media, the biological activities of the muteins were determined. Since the concentration of EPO in each case was adjusted to 10 mU/ml, the activities obtained here represent the specific bioactivity of each mutant hormone. Compared with the wild type EPO, the Tyr15-->Phe mutant and the Tyr49-->Phe mutant gave slightly lower activities, while the other two substitutions, Tyr145-->Phe and Tyr156-->Phe, resulted in production of about 1.5-fold higher activity clone (Fig. 4). But none of the mutants gave complete loss of the hormone's activity. Thus the result presented here is in clear contrast to the previous observation of Goldwasser. The inactivation of hormone's activity upon iodination they observed might be the result of local deformation in the tertiary structure of the hormone protein due to the chemical modification of tyrosine residues which are not directly involved in the binding to the hormone's receptor. Therefore, we conclude that any of the four tyrosine residues in erythropoietin is not essential in the biological activity of the hormone.

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