G00002

FUSION PROTEIN HAVING ENHANCED *IN VIVO* ACTIVITY OF ERYTHROPOIETIN

159159

TECHNICAL FIELD

The present invention relates to a fusion protein having enhanced *in vivo* activity of erythropoietin (EPO, below) that is a new medicine for the treatment of anemia. Specifically, the present invention relates to a fusion protein having highly enhanced *in vivo* half-life and activity of erythropoietin by fusion of EPO molecule with a certain peptide that has half-life elongation activity and is derived from the human body.

BACKGROUND ART

EPO, a glycoprotein having the molecular weight of 30,000 to 34,000, is a factor that stimulates production and differentiation of red blood cells. This protein acts by binding to receptors on erythrocyte precursor cells to result in increase of calcium ion concentration in a cell, increase of DNA biosynthesis, and stimulation for the formation of hemoglobin and the like. This EPO can be used for the treatment of anemia from renal failure, anemia of a premature baby, anemia from hypothyroidism, anemia from malnutrition, etc. The clinical use of recombinant human EPO is on the increase. However, such use may cause some inconvenience and high costs because it should be administered on the average three times a week due to its short half-life. Thus, if the *in vivo* activity of EPO is maintained for a long time, the administration frequency of EPO may be greatly decreased.

Efficacy of EPO is proportional to *in vivo* half-life thereof. It is known that *in vivo* half-life of EPO is correlated to the content of sialic acid that is located at the terminal of carbohydrate chains of EPO. Therefore, efficacy of EPO is highly dependent on the presence of carbohydrate chains. Since the forms of carbohydrates appear differently

depending on the kind of cells where EPO is expressed, the same glycoproteins may have different carbohydrate structure if they are expressed in different cells. Although it has been recently demonstrated that some bacteria can attach the carbohydrate chains, typical bacteria, for example *E. coli*, are known not to do. Proteins expressed in *E. coli* do not contain the carbohydrate chains, and thus, *E. coli*-derived EPO, which does not contain the carbohydrate chains, exhibits positive *in vitro* activity but no *in vivo* activity. It is because deglycosylated EPO is rapidly eliminated from the human body and has extremely short half-life. In conclusion, the carbohydrate chains play a very important role in the activity of EPO.

Many studies have been made to enhance the activity of EPO. The main approach is substitutions of some amino acids of EPO by mutagenesis on the EPO gene. For example, PCT/US94/09257, filed by Amgen and titled "Erythropoietin analogs," discloses a method to increase *in vivo* half-life of EPO by increasing the carbohydrate contents through mutagenesis. Also, an attempt to increase *in vivo* half-life of EPO was made by formation of EPO dimer. See, A. J. Sytkowski *et al.*, *J.B.C.* vol. 274, No. 35, pp24773-24778. Besides, another known method is to enhance *in vivo* activity of EPO by fusing new amino acids, peptides, or protein fragments with EPO molecule in the genetic engineering manner and increasing the carbohydrate content, i.e., sialic acid content of EPO. However, all amino acids, peptides, or heterogeneous protein fragments may not be used for this purpose. In most cases, such modifications may result in decrease or loss of inherent activity of protein and may cause a problem of antigenicity when used *in vivo*.

Although it is not related to EPO, fusion proteins or chimeric proteins have been studied, for example, for follicle stimulating hormone that is a kind of sex hormone. See, Furuhashi *et al.*, 1995, *Mol. Endocrinol.* However, the methods have not been applied to the industry since protein modifications using a genetic engineering method have many risks. That is, in most cases, the target protein may not be readily obtained without professional skills, and on the contrary, the inherent activity of protein may be decreased or lost by the addition or substitution of new amino acids.

DISCLOSURE OF THE INVENTION

The present inventors have extensively studied new methods for enhancing *in vivo* activity of EPO by fusing new amino acids, peptides, or protein fragments with EPO molecule, and so increasing the carbohydrate content thereof. As a result, the present inventors have discovered that a fusion protein of EPO obtained by fusing a carboxy terminal peptide (CTP, below) of thrombopoietin (TPO, below), a protein that already exists in the human body, with the carboxy terminal of EPO has highly enhanced *in vivo* half-life due to a lot of amino acids that increase glycosylation site without loss of the inherent activity of EPO, and does not cause any antigenicity when applied to the human body. Then, the present inventors have completed the present invention.

Therefore, the object of the present invention is to provide a fusion protein having enhanced *in vivo* activity of human EPO and containing CTP of TPO fused with human EPO at the carboxy terminal thereof.

Another object of the present invention is to provide a nucleic acid encoding the fusion protein, a recombinant vector containing the nucleic acid, and a host cell line transformed with the recombinant vector.

Further object of the present invention is to provide a process for preparation of the fusion protein having enhanced *in vivo* activity of human EPO by cultivating the transformed cell line.

First, the present invention relates to a fusion protein having enhanced *in vivo* activity of human EPO and containing CTP of TPO fused with human EPO at the carboxy terminal thereof. The CTP preferably comprises the amino acid sequence of SEQ ID No. 1 that corresponds to amino acids of positions 176 to 353 (LTP, below) or parts thereof, particularly the amino acid sequence of SEQ ID No. 2 that corresponds to amino acids of positions 337 to 353 (STP, below).

Particularly, the fusion protein according to the present invention may comprise the amino acid sequence of SEQ ID No. 3 or 4.

Second, the present invention relates to a nucleic acid encoding the fusion protein, a recombinant vector containing the nucleic acid, and a host cell line, preferably CHO (Chinese hamster ovary) cell, transformed with the recombinant vector.

Third, the present invention relates to a process for preparation of the fusion protein having enhanced *in vivo* activity of human EPO by cultivating the transformed cell line.

Below, the present invention will be explained in more detail.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b show the nucleotide and amino acid sequences of carboxy terminal peptides (LTP, STP) of TPO;

Figures 2aa and 2ab show the nucleotide and amino acid sequences of ELTP that is a fusion protein of EPO and the peptide LTP, and Figure 2b shows the nucleotide and amino acid sequences of ESTP that is a fusion protein of EPO and the peptide STP;

Figures 3a and 3b are schemes showing the procedures to prepare the expression vectors, pcDNA-ELTP and pcDNA-ESTP;

Figure 4 is an electrophoresis photograph of expressed ELTP and ESTP; and

Figure 5 is a graph showing the pharmacokinetic results of EPO, ELTP, and ESTP.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention comprises the steps of preparation and cloning of a gene of the desired fusion protein, construction of an expression vector containing the desired gene, transformation of an animal cell, expression, purification, and biological assay.

(1) Preparation of gene

EPO cDNA may be obtained by performing the conventional PCR technique (PCR PreMix Kit of Bioneer Co.) using the complementary primers of EPO cDNA terminals (EP1 and EP2) from cDNA library of human-derived fetal liver (Invitrogen Co.). TPO cDNA may be obtained using the complementary primers of TPO cDNA terminals (T1 and T2) according to the same manner.

EP1: ATGGGGGCACGAATGTCCTGCCTGGCTGG (SEQ ID No. 5)
EP2: GTCCCCTGTCCTGCAGGCCT (SEQ ID No. 6)
T1: ATGGATCTGACTGAATTGCTCCTC (SEQ ID No. 7)
T2: TTACCCTTCCTGAGACAGATTCTGGGA (SEQ ID No. 8)

EPO cDNA and TPO cDNA obtained by PCR are cloned to pGEM-T (Promega Co.), a cloning vector, respectively. The pGEM-EPO and pGEM-TPO are then sequenced, and used as a template for the following procedures.

LTP, a CTP gene of TPO used in the present invention, may be obtained by performing PCR using pGEM-TPO as a template and primers EL1 and T2.

EL1: GAGGCCTGCAGGACAGGGGACGCCCCACCCACCACAGCTGTCC (SEQ ID No. 9)

The primer EL1 contains a gene extended to the position of restriction enzyme *Stu*I, which is located near the 3' terminal of EPO, and a part of 5' terminal of LTP gene (amino acids of positions 176 to 353 of TPO) at the same time. On the other hand, PCR is performed using pGEM-EPO as a template and primers EP1 and EP2 to give EPO gene only. Thus obtained EPO and LTP genes are treated with restriction enzyme *Stu*I, respectively. Then, fragments of EPO and LTP genes are ligated and the ligation product is

used as a template in the PCR procedure using primers EP11 and L2 to give a gene fragment ELTP having about 1113 bps.

EP11: TAAGCTTATGGGGGTGCACGAATGT (SEQ ID No. 10)

L2: TGGATTCTTACCCTTCCTGAGACAGATTC (SEQ ID No. 11)

This gene is cloned to a cloning vector of pGEM-T and then sequenced (pGEM-ELTP, Figure 3a).

Further, the STP gene used in the present invention may be obtained by synthesis and self-priming PCR procedure. Synthesized gene fragments are the following ES1, S2, S3 and the above L2.

ES1: AGGGGAGGCCTGCAGGACAGGGGACCCTCTTCTAA (SEQ ID No. 12)

S2: GTGGGTGTAGGATGTGTTTAGAAGAGG (SEQ ID No. 13)

S3: TACACCCACTCCCAGAATCTGTCTC (SEQ ID No. 14)

1μℓ (50 pmole/μℓ) of each 4 gene fragments is taken and PCR is performed using high fidelity Taq system of BM Co.

The gene fragment of about 50 bps (STP gene) is identified in 1% agarose gel. This gene encodes the 17 amino acids (amino acids of positions 337 to 353) of the carboxy terminal of TPO (Figure 1).

PCR is performed using pGEM-EPO as a template and primers EP11 and EP2 to give EPO gene only. This EPO gene and the STP gene are used as templates at the same time and primers EP11 and L2 are used in a PCR using the high fidelity Taq system of BM Co. to give the desired fusion gene, ESTP gene, of about 630 bps. This gene is cloned to pGEM-T, a cloning vector, and then sequenced (pGEM-ESTP, Figure 3b).

(2) Construction of expression vector

pcDNA3.1 vector of Invitrogen Co. is used as an expression vector.

The pcDNA3.1 vector is treated with restriction enzymes *Hin*dIII and *Bam*HI to make a linear vector, and pGEM-ELTP and pGEM-ESTP are also treated with the same restriction enzymes, *Hin*dIII and *Bam*HI, respectively. The digested pcDNA3.1, ELTP, and ESTP genes are isolated using Qiagen extraction kit on agarose gel. After each ligation of the genes, the ligation product is introduced into *E. coli* NM522. Plasmids are isolated from the colonies that have been formed from cultivation of the transformed *E. coli* in LB-ampicillin plate overnight, and are treated with restriction enzymes, *Hin*dIII and *Bam*HI. Colonies having ELTP or ESTP gene are then screened by 1% agarose gel electrophoresis. These plasmids are designated as pcDNA-ELTP and pcDNA-ESTP, respectively (Figures 3a and 3b).

(3) Transformation of CHO cell and expression

CHO cells (DG44) are cultivated to a confluency of 40~80% (1~ 4×10^5 cells/60mm dish) in a 60mm cell culture dish. 3㎕ of superfection reagent (BM Co.) and 97㎕ of cell culture medium (a-MEM with media, no serum, no antibiotics) are mixed well, and pcDNA-ELTP (=0.1㎍/㎕, about 2㎍) and vector pLTRdhfr26 (ATCC37295, 0.2㎍) having dhfr gene are added thereto. The mixture is reacted for 5~10 minutes at room temperature and then added to the cells as prepared above. After one day, the medium is refreshed with a medium containing G418 in an amount of 500㎍/㎖ (a-MEM without media, 10% FBS). The medium is then refreshed with the G418 medium containing 500㎍/㎖ of G418 for 7~10 days, during which cells without G418 resistance gene and negative control cells are completely killed. Selected cells on G418 medium are cultivated sufficiently and an expressed ELTP protein is identified using EPO ELISA kit of BM Co. The same process is applied to ESTP expression vector and then an expressed ESTP fusion protein is also identified in the same manner.

(4) Purification of the expressed ELTP and ESTP

Affinity resin for isolation and purification is prepared using anti-EPO monoclonal antibody (R&D Co.) as follows:

0.3g of CNBr-activated Sepharose 4B is swelled for 20 minutes in 1mM HCl, and the resin is moved to a column and washed with 1mM HCl. The resin is washed with 4ml of coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3), immediately mixed with anti-EPO monoclonal antibody (500μg/vial) contained in 4ml of coupling buffer, and reacted for 2 hours at room temperature with stirring the tube. Refreshed with blocking buffer (0.2M glycine, pH 8.0), the resin is reacted at room temperature for 2 hours with stirring the tube. Then, the resin is washed with 6.5ml of coupling buffer, 6.5ml of acetate buffer (0.1M acetic acid, 0.5M NaCl, pH 4), and 6.5ml of coupling buffer in the order. A column is prepared using such obtained resin and purification is conducted as described below.

Cells are cultivated in serum free medium for one day, and the medium is concentrated to about 5 folds using Centriprep (Millipore MWCO 10,000). This concentrate is loaded to a column equilibrated in advance with PBS at a flow rate of 20ml/hr, and washed again with PBS. Elution buffer (0.1M glycine, pH 2.8) is applied to the column and the eluent is immediately titrated with 1M Tris to pH7.5. The purity is at least 97% when analyzed by SDS-PAGE and silver staining (Figure 4).

(5) Activity measurement by bioassay method

In the bioassay test using mouse spleen cells treated with phenylhydrazine, the ELTP and ESTP, which are expressed and properly purified, show higher activity than EPO. It demonstrates that the fused carboxy terminals in the ELTP and ESTP may not inhibit activity of EPO itself.

(6) Pharmacokinetic experiments

Pharmacokinetic experiments are performed to mice to determine whether the prepared ELTP and ESTP actually have prolonged *in vivo* half-life. The candidate substance is intravenously administered to 4 mice in an amount of 20 units/mouse. In order to identify the time-lapse concentration in blood, blood is taken from the mice at an

interval of 30 minutes and concentrations are measured using EIA kit of Boehringer Mannheim Co. In the pharmacokinetic experiments to mice, the candidate substances, ELTP and ESTP, show far longer half-life than the control EPO (Figure 5).

The present invention will be more specifically explained in the following examples. However, it should be understood that the following examples are intended to illustrate the present invention but not to limit the scope of the present invention in any manner.

Example 1: Preparation of gene

EPO cDNA was obtained by performing the conventional PCR (PCR PreMix Kit of Bioneer Co.) using the primers EP1 and EP2 that are complementary to the terminals of EPO cDNA, in an amount of 50 pmole, respectively, from cDNA library of human-derived fetal liver (Invitrogen Co.). TPO cDNA was obtained using the primers T1 and T2, complementary to the terminals of TPO cDNA, according to the same manner. Total 30 cycles of PCR were performed using high fidelity Taq system of BM Co. under the condition of 52°C for 40 seconds for annealing, 72°C for 55 seconds, and 94°C for 20 seconds to give EPO cDNA and TPO cDNA. They were cloned to pGEM-T (Promega Co.), a cloning vector, respectively. That is, the PCR products were eluted from 1% agarose gel and ligated into pGEM-T, which was then introduced into *E. coli* NM522. The transformed *E. coli* was cultivated overnight in LB-ampicillin plate containing X-gal/IPTG. Plasmids were purified from the white colonies and treated with restriction enzymes, *Sac*I and *Sac*II , to screen the colonies containing the respective cDNAs. The vectors obtained at this stage were designated as pGEM-EPO and pGEM-TPO, which were then sequenced, and used as templates for the following procedures.

LTP, CTP gene of TPO used in the present invention, was obtained by performing total 30 cycles of PCR using the high fidelity Taq system of BM Co. and using pGEM-TPO as a template and primers, EL1 and T2 (50 pmole), under the condition of 50°C for 40 seconds for annealing, 72°C for 45 seconds, and 94°C for 20 seconds. The primer EL1 contains a gene from the position of restriction enzyme *Stu*I, which is located near the 3'

terminal of EPO, to the 3' terminal, and a part of 5' terminal of LTP gene at the same time. A gene fragment of about 534 bps was identified on 1% agarose gel (LTP gene). This gene encodes the 178 amino acids of the carboxy terminal of TPO (amino acids of positions 176 to 353) (Figure 1).

On the other hand, PCR was performed using pGEM-EPO as a template and primers EP1 and EP2 to give EPO gene only.

Thus obtained EPO and LTP genes were treated with restriction enzyme *StuI*, respectively. Then, fragments of EPO and LTP genes were purified by Qiagen extraction kit and ligated with a ligase. The ligation product of EPO and LTP genes was used as a template in total 30 cycles of PCR procedure using primers EP11 and L2 (50 pmole) and using the high fidelity Taq system of BM Co. under the condition of 55°C for 40 seconds for annealing, 72°C for 60 seconds, and 94°C for 20 seconds to give the desired fusion gene of ELTP having about 1113 bps. This gene was cloned to a cloning vector of pGEM-T in the same manner as above and then sequenced (pGEM-ELTP, Figure 3a).

STP gene was obtained by synthesis and self-priming PCR procedure. Synthesized DNA fragments were ES1, S2, S3 and L2. 1μℓ (50 pmole/μℓ) of each of the 4 DNA fragments was taken and total 15 cycles of PCR were performed using the high fidelity Taq system of BM Co. under the condition of 55°C for 40 seconds for annealing, 72°C for 40 seconds, and 94°C for 20 seconds. A gene fragment of about 50 bps was identified on 1% agarose gel (STP gene). This gene encodes the 17 amino acids of the carboxy terminal of TPO (amino acids of positions 337 to 353) (Figure 1).

PCR was performed using pGEM-EPO as a template and primers EP11 and EP2 in the same manner as above to give EPO gene only. This EPO gene and the STP gene were used as templates at the same time and primers of EP11 and L2 were used in total 30 cycles of PCR using high fidelity Taq system of BM Co. under the condition of 58°C for 40 seconds for annealing, 72°C for 50 seconds, and 94°C for 20 seconds to give the desired fusion gene, ESTP gene, of about 630 bps. This gene was cloned to pGEM-T, a cloning vector, and then sequenced (pGEM-ESTP, Figure 3b).

Example 2: Construction of expression vectors pcDNA-ELTP and pcDNA-ESTP

pcDNA3.1 vector of Invitrogen Co. was used as an expression vector. ELTP and ESTP genes that are cloned to pGEM-T vector contain *Hin*dIII and *Bam*HI recognition sites at terminals, respectively. pcDNA3.1, pGEM-ELTP and pGEM-ESTP were treated with restriction enzymes, *Hin*dIII and *Bam*HI, respectively. The linearized pcDNA3.1, ELTP, and ESTP genes were isolated using Qiagen extraction kit on agarose gel. After ligation of pcDNA3.1 with ELTP or ESTP, the ligation product was introduced into *E. coli* NM522. Plasmids were isolated from the colonies that had been formed from cultivation of the transformed *E. coli* in LB-ampicillin plate overnight, and were treated with restriction enzymes, *Hin*dIII and *Bam*HI. Colonies having ELTP or ESTP gene were then screened by 1% agarose gel electrophoresis. These plasmids were designated as pcDNA-ELTP and pcDNA-ESTP, respectively (Figure 3).

Example 3: Transformation of CHO cell and expression

CHO cells (DG44) were cultivated to a confluency of 40~80% (1~ 4×10^5 cells/60mm dish) in a 60mm cell culture dish. 3μℓ of superfection reagent (BM Co.) and 97μℓ of cell culture medium (a-MEM with media, no serum, no antibiotics) were mixed well, and plasmid pcDNA-ELTP (=0.1μg/μℓ, about 2μg) and vector pLTRdhfr26 (ATCC37295, 0.2μg) having dhfr gene were added thereto. The mixture was reacted for 5~10 minutes at room temperature and then added to the cells as prepared above. After one day, the medium was refreshed with a medium containing G418 in an amount of 500μg/Mℓ (a-MEM without media, 10% FBS). The medium was then refreshed with the G418 medium containing 500μg/Mℓ of G418 for 7~10 days, during which cells without G418 resistance gene and negative control cells were completely killed. Selected cells on G418 medium were cultivated sufficiently and an expressed ELTP protein was identified using EPO ELISA kit of BM Co. The same process was applied to pcDNA-ESTP.

Example 4: Purification of the expressed fusion proteins

Affinity resin for isolation and purification was prepared using anti-EPO monoclonal antibody (R&D Systems Co.) as follows:

0.3g of CNBr-activated Sepharose 4B was swelled for 20 minutes in 1mM HCl, and the resin was moved to a column and washed with 1mM HCl. The resin was washed with 4ml of coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3), immediately mixed with anti-EPO monoclonal antibody (500μg/vial) contained in 4ml of coupling buffer in a tube, and reacted for 2 hours at room temperature with stirring the tube. Refreshed with blocking buffer (0.2M glycine, pH 8.0), the resin was reacted at room temperature for 2 hours with stirring the tube. Then, the resin was washed with 6.5ml of coupling buffer, 6.5ml of acetate buffer (0.1M acetic acid, 0.5M NaCl, pH 4), and 6.5ml of coupling buffer in the order. A column was prepared using such obtained resin and purification was conducted as described below.

Cells were cultivated in serum free medium for one day, and the medium was concentrated to about 5 folds using Centriprep (Millipore, MWCO 10,000). This concentrate was loaded to a column equilibrated in advance with PBS at a flow rate of 20ml/hr, and washed again with PBS. Elution buffer (0.1M glycine, pH 2.8) was applied to the column and the eluent was immediately titrated with 1M Tris to pH 7.5. SDS-PAGE results of the purified ELTP and ESTP are shown in Figure 4. The purity was at least 97% when analyzed by SDS-PAGE and silver staining.

Example 5: Activity measurement by bioassay method

Phenylhydrazine was injected to a mouse once a day for 2 days at a dose of 60mg/kg. After 3 days, enlarged spleen was separated and ground with a homogenizer to obtain spleen cells. The spleen cells were diluted to $6x10^6$ cells/ml and 100μl of the cell solution was introduced into each well of a 96 well plate. 0~500 mU/ml of authentic EPO and 100 mU/ml of the expressed fusion protein were added to each well, and the plate was allowed to stand at 37°C for 22 hours in a CO_2 incubator. 50μl of dimethyl ^{3}[H]-thymidine (20μCi/ml) was added to each well, further reacted for 2 hours in the same incubator, and the reaction solution was then adsorbed onto a glass filter (Nunc 1-73164) per each well.

The filters were washed with physiological saline 3 times and the amount of radioactivity of each filter was measured using ß counter. The activities of the fusion proteins, ELTP and ESTP were shown to be comparable to or higher than that of authentic EPO, which demonstrated that the carboxy terminals fused into EPO did not inhibit activity of EPO itself.

Example 6: Pharmacokinetic experiments

Pharmacokinetic experiments were performed to mice to determine whether the prepared candidate substance actually has prolonged *in vivo* half-life. The fusion protein purified according to the process of Example 5 was intravenously administered to 4 mice in an amount of 20 units/mouse. In order to identify the time-lapse concentration in blood, blood was taken from the mice at an interval of 30 minutes and concentrations were measured using EIA kit of Boehringer Mannheim Co. The results are shown in Figure 5. As can be seen from Figure 5, ELTP and ESTP show about three times longer half-life than the control, EPO, respectively (half-life of EPO was 22 minutes, that of ELTP was 60 minutes, and that of ESTP was 57 minutes).

INDUSTRIAL APPLICABILITY

The fusion proteins according to the present invention have highly increased *in vivo* half-life of EPO due to the presence of amino acids that increase the carbohydrate chains without influencing the inherent activity of EPO. Further, the fusion proteins do not cause any problem of antigenicity when applied to the human body since the fused peptide, LTP or STP, is a peptide that already exists in the body.

【Sequence List】

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35 40 45

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100 105 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
115 120 125

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145 150 155 160

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<223> Primer T1 having the nucleotide sequence complementary to the terminal sequence of TPO cDNA

<400> 7

atggatctga ctgaattgct cctc

24

<210> 8

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer T2 having the nucleotide sequence complementary to the terminal sequence of TPO cDNA

<400> 8
ttacccttcc tgagacagat tctggga
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<210> 9
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer EL1 for PCR to obtain the desired gene LTP

<400> 9
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43

<210> 10
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer EP11 for PCR to obtain the desired fusion gene ELTP and ESTP

<400> 10
taagcttatg ggggtgcacg aatgt
25

<210> 11

<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer L2 for PCR to obtain the desired fusion gene ELTP and ESTP

<400> 11
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<210> 12
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer ES1 for PCR shuffling to obtain the desired gene STP

<400> 12
aggggaggcc tgcaggacag gggaccctct tctaa 35

<210> 13
<211> 27
<212> DNA
<213> Artificial Sequence

<220>

<223> Primer S2 for PCR shuffling to obtain the desired gene STP

<400> 13
gtgggtgtag gatgtgttta gaagagg
27

<210> 14
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer S3 for PCR shuffling to obtain the desired gene STP

<400> 14
tacacccact cccagaatct gtctc
25

CLAIMS

1. A fusion protein wherein a carboxy terminal peptide (CTP) of thrombopoietin (TPO) is fused with the carboxy terminal of human erythropoietin (EPO).

2. The fusion protein of claim 1 wherein the carboxy terminal peptide comprises the amino acids of positions 176 to 353 of TPO, the sequence of which is described in SEQ ID No. 1, or parts thereof.

3. The fusion protein of claim 2 wherein the carboxy terminal peptide comprises the amino acids of positions 337 to 353 of TPO, the sequence of which is described in SEQ ID No. 2.

4. Nucleic acid encoding the fusion protein according to any one of claims 1 to 3.

5. A process for preparation of a fusion protein having enhanced *in vivo* activity of human EPO comprising cultivation of a host cell line transformed with the recombinant vector containing the nucleic acid according to claim 4.