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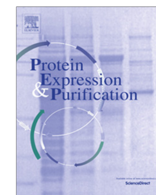

Engineering of erythropoietin receptor for use as an affinity ligand

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ABSTRACT

Recombinant human erythropoietin receptor (rhEPOR) has applicability as an affinity ligand for purification of recombinant human erythropoietin (rHuEPO) because of its specific binding to rHuEPO. For application of rhEPOR as a ligand for purification of rHuEPO, soluble rhEPOR was expressed in the periplasm of *Escherichia coli* and engineered by directed evolution through random mutagenesis and integration of mutations. From the screening of random mutagenesis, we identified an amino acid mutation (H114Y) contributing to rHuEPO binding and four amino acid mutations (R76S, A132D, A162D, and C181Y) contributing to expression of soluble rhEPOR. However, the rHuEPO that binds to engineered rhEPOR having H114Y mutation is difficult to dissociate from the engineered rhEPOR. Therefore, H114Y mutation was not suitable for the construction of the rhEPOR ligand. As a rhEPOR ligand, engineered rhEPOR containing four amino acid mutations (EPORm4L) was constructed by integration of mutations except for H114Y. The expression of EPORm4L (127 mg l^{-1} of culture medium) was markedly increased in comparison with wild-type rhEPOR (2 mg l^{-1} of culture medium). Small-scale affinity chromatography demonstrated that EPORm4L worked as an affinity ligand for purification of rHuEPO.

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Introduction

Human erythropoietin (EPO)[1] is a glycoprotein hormone that regulates erythropoiesis [1]. Recombinant human EPO (rHuEPO) and analogous erythropoiesis-stimulating agents (e.g., hyperglycosylated analog of rHuEPO) are used as therapeutic agents for treating diseases such as anemia [2]. For the production of these therapeutic agents related to rHuEPO, transfected Chinese hamster ovary (CHO) cells are generally used. To purify these therapeutic agents, a multistep purification protocol including a capture step of ion-exchange or reversed-phase chromatography, followed by affinity chromatography and the additional steps of size exclusion and ion-exchange chromatography, is required [3]. Recently, several affinity ligands for purification of rHuEPO, for example monoclonal antibody, peptide, and de novo synthetic ligands, have been reported [3–5].

From the point of view of specific binding to EPO, it was thought that human EPO receptor (EPOR) might be suitable as an affinity ligand for rHuEPO purification. In erythropoiesis, EPO binds and activates homodimeric EPOR on the surface of erythroid progenitor cells [6]. Human EPOR is a glycoprotein of approximately 59 kDa with an added *N*-linked carbohydrate chain, and consists of an extracellular region that binds to EPO, a transmembrane region, and a cytoplasmic region [2,7]. The recombinant protein consisting of the extracellular region of human EPOR (recombinant human EPOR, rhEPOR) might become an affinity ligand for purification of rHuEPO. Therefore, we tackled the development of an affinity ligand from rhEPOR in this study.

Previously, we reported engineering of recombinant human FcγRI, which is the receptor of IgG, by directed evolution [8]. To develop an affinity ligand from rhEPOR, we applied the protein engineering technique described in our previous report to the engineering of rhEPOR.

Materials and methods

Construction of vectors

To construct the pET-MalE21 vector, PCR was performed using PrimeSTAR HS DNA Polymerase (Takara Bio, Shiga, Japan) and the pET-p7-MFcR vector [9] as a template and primers 1 and 2 (Supplementary Table S1). This PCR product digested by XbaI and NcoI was inserted into the XbaI–NcoI site of the pET-26b vector (Novagen, Merck KGaA, Darmstadt, Germany).

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[1] *Abbreviations used:* EPO, erythropoietin; rHuEPO, recombinant human erythropoietin; EPOR, erythropoietin receptor; rhEPOR, recombinant human erythropoietin receptor; CHO, Chinese hamster ovary.

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The EPORwt gene (encoding the extracellular domain of EPOR) (DDBJ accession number: AB937115) was constructed by PCR using the primers listed in Supplementary Table S1. First, PCR was performed using EPOR cDNA (human) (OriGene Technologies, Rockville, MD, USA) as a template and primers 3 and 4. Second, PCR was performed using EPOR cDNA and primers 5 and 6. Third, PCR was performed using the purified first and second PCR products and primers 3 and 7. Fourth, the PCR product digested by NcoI and HindIII was inserted into the NcoI-HindIII site of the pET-MalE21 vector. This constructed vector was designated as the EPORwt expression vector. Cells of *Escherichia coli* BL21 (DE3) or *E. coli* NiCo21 (DE3) (New England Biolabs, Ipswich, MA, USA) were transformed with the EPORwt expression vector.

Enzyme-linked immunosorbent assay (ELISA) for detection of binding between rhEPOR and rHuEPO (ELISA [binding assay])

A MaxiSorp 96-well plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) was coated with Human Erythropoietin R Antibody (R&D Systems, Minneapolis, MN, USA) (0.1 μg well^{-1}) in 50 mM Tris–HCl buffer (pH 8.0) overnight at 4 °C. Blocking was performed with TBS-B buffer (20 mM Tris–HCl, 137 mM NaCl, 2.68 mM KCl, 0.5% (w/v) bovine serum albumin, pH 7.4) for 2 h at 30 °C. After the wells had been washed with wash buffer (0.05% [w/v] Tween 20, 150 mM NaCl, 20 mM Tris–HCl, pH 8.0), sample solutions containing rhEPOR (40 ng well^{-1}) were added to the wells and incubated for 1.5 h at 30 °C. After washing, biotinylated rHuEPO (rHuEPO [Erythropoietin, Human, Recombinant, CHO Cells, Calbiochem, Merck KGaA, Darmstadt, Germany] was biotinylated using EZ-Link Sulfo-NHS-Biotin [Pierce, Thermo Scientific, Rockford, IL, USA]) (40 ng well^{-1}) in TBS-B buffer was added to the wells and incubated for 1.5 h at 30 °C. After washing, horseradish peroxidase (HRP)-streptavidin conjugate (1:2500 dilution, Invitrogen, Life Technologies, Carlsbad, CA, USA) was added to the wells and incubated for 1.5 h at 30 °C. After washing, TMB microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) was added to the wells for color development, and the optical density was read at a wavelength of 450 nm.

ELISA for determination of soluble rhEPOR concentration (ELISA [rhEPOR])

A MaxiSorp 96-well plate was coated with Human Erythropoietin R Antibody (0.1 μg well^{-1}) in 50 mM Tris–HCl buffer (pH 8.0) overnight at 4 °C. Blocking was performed with TBS-B buffer. After washing, sample solutions were added to the wells, and incubated for 1.5 h at 30 °C. After washing, HRP-conjugated rabbit anti-6His antibody (Bethyl Laboratories, Montgomery, TX, USA) was added to the wells and incubated for 1.5 h at 30 °C. After washing, TMB microwell peroxidase substrate was added to the wells for color development, and optical density was read at a wavelength of 450 nm. The quantity of rhEPOR was calculated from the ELISA data with glycosylated rhEPOR (grhEPOR) (Sino Biological, Beijing, China, catalog number: 10707-H08H) as a standard.

ELISA for determination of rHuEPO concentration (ELISA [rHuEPO])

A MaxiSorp 96-well plate was coated with monoclonal antibody to erythropoietin (clone Epo2, Acris Antibodies, Herford, Germany) (0.2 μg well^{-1}) in 50 mM Tris–HCl buffer (pH 8.0) overnight at 4 °C. Blocking was performed with TBS-B buffer. After washing, sample solutions were added to the wells and incubated for 1.5 h at 30 °C. After washing, biotinylated monoclonal antibody to erythropoietin (antibody [clone Epo1, Acris Antibodies] was biotinylated by using EZ-Link Sulfo-NHS-Biotin) (380 ng well^{-1}) in TBS-B buffer was added to the wells and incubated for 1.5 h at 30 °C. After washing, HRP-streptavidin conjugate was added to the wells and incubated for 1.5 h at 30 °C. After washing, TMB microwell peroxidase substrate was added to the wells for color development, and optical density was read at a wavelength of 450 nm. The quantity of rHuEPO was calculated from the ELISA data.

Random mutational library

Random mutations were introduced by error-prone PCR in accordance with the method of Asaoka et al. [8] except for the template, forward primer, and reverse primer. Primers 3 and 7 (Supplementary Table S1) were used as forward and reverse primers, respectively. Error-prone PCR for the first random mutational library used the EPORwt expression vector as a template. Error-prone PCR for the second random mutational library used the EPORm1 expression vector as a template. These PCR products were digested with NcoI and HindIII and inserted into the pET-MalE21 vector. Cells of *E. coli* BL21 (DE3) were transformed with these constructed vectors, and random mutational libraries were thus constructed.

Screening of the random mutational library

Expression was essentially performed in accordance with the method of Asaoka et al. [8]. Each transformant was cultured aerobically overnight at 37 °C in Luria–Bertani (LB) medium containing 50 μg ml^{-1} kanamycin. Each overnight culture (20 μl) was inoculated into 500 μl of LB medium containing 50 μg ml^{-1} kanamycin, 0.3% (w/v) glycine, and 0.05 mM isopropyl-b-D(−)-thiogalactopyranoside (IPTG). The expressed randomly mutated rhEPOR was secreted into the culture medium by the addition of glycine [8]. It was then aerobically incubated by shaking (1200 rpm, Deep Well Maximizer, Taitec, Saitama, Japan) at 20 °C for 24 h in 96-well deep plates (Greiner Bio-one, Monroe, NC, USA). After the incubation, supernatants containing randomly mutated rhEPOR were obtained by centrifugation (2500 rpm, 20 min, 4 °C). For the first random mutational library, each supernatant was diluted 10 times with TBS-B buffer. Subsequently, the binding activity of each randomly mutated rhEPOR to rHuEPO was determined by ELISA (binding assay), and candidates for mutated rhEPOR with high expression or strong binding to rHuEPO were selected. For the second random mutational library, each supernatant underwent acid treatment in the following steps: (1) mixing 20 μl of supernatant with 30 μl of 0.1 M citrate buffer (pH 3.0), (2) incubation for 1 h at 30 °C, and (3) neutralization by adding 20 μl of 1 M Tris–HCl buffer (pH 7.4). Each acid-treated supernatant was diluted two times with TBS-B buffer. Subsequently, binding activity of each randomly mutated rhEPOR to rHuEPO was determined by ELISA (binding assay). Candidates for mutated rhEPOR with high expression or strong binding to rHuEPO were selected.

Construction of engineered rhEPOR (integration of mutations)

Engineered rhEPORs (EPORm3a, EPORm3b, EPORm4, and EPORm5) and EPORm4L were constructed by site-directed mutagenesis to EPORm2 and EPORwt, respectively. Genes of these engineered rhEPORs were constructed by PCR using the primers listed in Supplementary Table S1. The constructed gene was inserted into the pET-MalE21 vector at the NcoI-HindIII site. The cells of *E. coli* BL21 (DE3) were transformed with the constructed expression vectors of EPORm3a, EPORm3b, EPORm4, and EPORm5. The cells of *E. coli* NiCo21 (DE3) were transformed with the constructed expression vector of EPORm4L. The DDBJ accession numbers for EPORm5 and EPORm4L are AB937116 and AB937117, respectively.

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