An Erythropoietin Fusion Protein Comprised of Identical Repeating Domains Exhibits Enhanced Biological Properties

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Arthur J. Sytkowski, Elizabeth Dotimas Lunn, Mary A. Risinger, and Kerry L. Davis

From the Laboratory for Cell and Molecular Biology, Division of Hematology and Oncology, Beth Israel Deaconess Medical Center, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

The hematopoietic growth factor erythropoietin (Epo) initiates its intracellular signaling cascade by binding to and inducing the homodimerization of two identical receptor molecules. We have now constructed and expressed in COS cells a cDNA encoding a fusion protein consisting of two complete human Epo domains linked in tandem by a 17-amino acid flexible peptide. On SDS-polyacrylamide gel electrophoresis, the Epo-Epo fusion protein migrated as a broad band with an average apparent molecular mass of 76 kDa, slightly more than twice the average apparent molecular mass of Epo, 37 kDa. Enzymatic N-deglycosylation resulted in an Epo-Epo species that migrated on SDS-polyacrylamide gel electrophoresis as a narrow band with an average apparent molecular mass of 39 kDa. The specific activity of the Epo-Epo fusion protein in vitro (1,007 IU/μg; 76 IU/μmol) was significantly greater than that of Epo (352 IU/μg; 13 IU/μmol). Moreover, secretion of Epo-Epo by COS cells was 8-fold greater than that of Epo. Subcutaneous administration of a single dose of Epo-Epo to mice resulted in a significant increase in red blood cell production within 7 days. In contrast, administration of an equivalent dose of conventional recombinant Epo was without effect. The pharmacokinetic behavior of Epo-Epo differed significantly from that of Epo. The results suggest that Epo-Epo may have important biological and therapeutic advantages.

Recombinantly produced proteins are gaining wide use as injectable pharmaceuticals to treat a variety of deficiencies and diseases. A problem encountered in their use is the frequency with which injections must be made in order to maintain a therapeutic level in the circulation. One means of increasing the plasma half-life of injected proteins is chemical conjugation with polyethylene glycol (“pegylation”) (1). Although apparent success has been achieved with some proteins (2), this method can sometimes alter protein structure, reduce biological activity, or cause unanticipated changes in specificity and function (3–5). We hypothesized that an alternative approach would be the production of a multivalent molecule consisting of two or more biologically active units of the same protein. We speculated that these molecules would exhibit an increased plasma half-life and would also possess enhanced activity due to facilitated binding of the repeating units to their cognate receptors and to amplification of the intracellular signaling pathways. We and others have shown previously that such molecules could be produced by chemical cross-linking (6, 7). However, a fusion protein with two human erythropoietin (Epo) domains linked by three to seven amino acids exhibited reduced in vitro activity compared with the wild-type monomer (8).

We now report the production of a recombinant fusion protein consisting of two complete human Epo molecules in tandem separated by a 17-amino acid linker. Both Epo domains of the fusion protein are equally biologically active. Importantly, the protein has substantially enhanced potency and efficacy over conventional recombinant Epo in vitro and in vivo and is efficacious after a single subcutaneous injection.

EXPERIMENTAL PROCEDURES

Construction of Epo-Epo Fusion Protein cDNA—Two different Epo cDNA constructs were produced by amplifying pSV2-Epo (9) and then ligated to form the Epo-Epo fusion protein cDNA. The initial preceding (Domain A) Epo DNA strand (Epo A1) was produced by amplifying pSV2-Epo using primers EpA5 and EpA3-3 (see below). The 3′ end was sequentially extended by amplifying with EpA3-4 and EpA3-5. Epo A2 was produced by amplifying Epo A1 with primers EpA5 and EpA3-4, and Epo A3 was produced by amplifying Epo A1 with primers EpA5 and EpA3-5. The succeeding (Domain B) Epo DNA strand (Epo B) was obtained by amplification of pSV2 Epo using EpB5 and EpB3. Gel-purified Epo A3 and Epo B were ligated into the cloning vector pCR-blunt (InVitrogen) using a vector/insert ratio of 1:10. Kanamyacin-resistant colonies (50 μg/ml) were plucked, and positive clones were identified by restriction digest analysis using Bgl II. The resulting constructs were designated Epo A (blunt and Epo B) blunt. The primer sequences were as follows: (a) EpA5, 5′-AGCCGCGGATGATGGGGTGCAGC; (b) EpA3-3-3, 3′-CCAGATCCACCCCGGGCTCTGTCCCTTCGTCGAG; (c) EpA3-4, 3′-GGCCAGCGGATCCACCCCCGCCACGATCACCGCCGCC; (d) EpA3-5, 5′-TGGTGGGGCAGTACTGCCGCCGCCAC; (e) EpB5, 5′-CCGCCGATCTGGCCACCGCCAGCCTCTGTCGAGC; and (f) EpB3, 5′-CAGGGTGACACACCTGGTGACCTGCAC.

Epo A/blunt was digested with ScaI and XhoI, whereas Epo B/blunt was digested with ScaI and BamHI. Gel-purified Epo A/blunt and Epo B/blunt digests were ligated in a 1:1 molar ratio. The resulting constructs were gel-purified and then ligated into expression vector pCDNA3.1(−/−)(InVitrogen) that had been digested previously with BamHI and XhoI and gel-purified. Ampicillin-resistant colonies (10 μg/ml) were plucked, and positive clones were identified by restriction digest analysis using NcoI.

Polymerase Chain Reaction—The reactions (50 μl) contained 0.5 μM 5′ or 3′ primer; 10 ng of pSV2-Epo; 200 μM dATP, dUTP, dTTP, and dGTP; 20 mM Tris-HCl, pH 8; 2 mM MgCl2; 10 mM KCl; 6 mM (NH4)2SO4; 0.1% Triton X-100; 10 g/ml nuclease-free bovine serum albumin; and 2.5 units/plaque-forming unit DNA polymerase (Stratagene). They were

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† To whom correspondence should be addressed: Laboratory for Cell and Molecular Biology, Division of Hematology and Oncology, Beth Israel Deaconess Medical Center, One Deaconess Rd., 21-27 Burlington, Boston, MA 02215. Tel.: 617-632-9980; Fax: 617-632-0401; E-mail: asytkows@caregroup.harvard.edu.

‡ The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Epoκ, wild-type erythropoietin; ELISA, enzyme-linked immunosorbent assay; TBST, 20 mM Tris-HCl, 0.5% NaCl, 0.5% Tween 20.
subjected to 25 cycles of (a) 1-min denaturation at 95 °C, (b) 1-min annealing at 58 °C, and (c) 1-min extension at 72 °C.

Ligation and Transformation Reactions—Ligation reactions for pCR-blunt contained 25 ng of vector and a 10-fold molar excess of either Epo A3 or Epo B. 10-μl reactions contained 6 μl Tris-HCl, pH 7.5, 6 μM MgCl2, 2 μg BSA, 1 mM each dNTP, 7.5 μM mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine, and 4 Weiss units of T4 DNA ligase (InVitrogen). Incubations were carried out for 1 h at 16 °C. pcDNA 3.1(−) ligations contained a vector-insert ratio of 5:1, and the reaction conditions described above were used. 50 μL of One Shot TOP10 competent cells (InVitrogen) were transformed with 2 μl of each reaction mixture according to the manufacturer’s procedure.

Construction of Epo-Epo R103A Mutants—An Epo mutant with arginine 103 replaced by alanine, psV2-Epo 103 (R103A) (10) was spliced into Epo Ablunt to produce Epo 103A/ablunt. Epo 103B/ablunt was produced by ampling psV2-Epo103 using primers EpoB-51 and EpoB3. Restriction sites used to insert the mutant strand were BglI and AccI for Epo 103A/ablunt. The procedure for ligating Epo A, Epo B, Epo 103A, and Epo 103B was described above. Three mutant fusion constructs were produced: (a) Epo A–Epo 103B (pcDNA 3.1(−)), (b) Epo A–Epo 103B (pcDNA 3.1(−)), and (c) Epo A–Epo 103B (pcDNA 3.1(−)), resulting in fusion proteins EpoA–Epo103A, EpoR103A–EpoR103A, and EpoR103A–EpoR103A, respectively. The mutant Epo 103 was also spliced into pcDNA3.1(−) in the ECoNI restriction sites.

RNA Extraction and Northern Blot Analysis—Total RNA was prepared using TRIzol reagent (Life Technologies, Inc.). The manufacturer’s protocol was followed, and the RNA obtained was separated on 1.2% agarose in 40–50 mM Tris-borate, 192 mM glycine, and 10% methanol. Membranes were then rinsed twice with distilled water and incubated overnight at 4 °C in TBST and washing were again carried out as described above, plus two additional washes for 5 min each. Protein bands recognized by the primary antibody were detected using a luminescence kit (ECL, Amersham Pharmacia Biotech). The ELISA (Genzyme) was calibrated with recombinant human Epo.

Deglycosylation of the Epo-Epo Fusion Protein—SDS (0.5%) and 5% β-mercaptoethanol were added to a COS1 cell culture supernatant containing the Epo-Epo fusion protein, and the solution was heated at 100 °C for 2 min. After cooling, 0.5 volume of 40–50 μM nitrocyclulose membranes in 25 mM Tris-HCl, 192 mM glycine, and 10% methanol. Membranes were then rinsed twice with distilled water and incubated overnight at 4 °C in TBST and 10% nonfat dry milk, pH 7.5. The membranes were rinsed twice with TBST, washed once with TBST for 15 min and washed twice with TBST for 5 min each. The membranes were then incubated with anti-erythropoietin monoclonal antibody AE-7A5 (11) (Genzyme); 0.7 μg/ml TBST and 5% nonfat dry milk for 1 h at 23 °C. Rinsing and washing were carried out as described above, followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel) diluted 1:1000 in TBST and 5% nonfat dry milk for 1 h at 23 °C. Rinsing and washing were again carried out as described above, plus two additional washes for 5 min each. Protein bands recognized by the primary antibody were detected using a luminescence kit (ECL, Amersham Pharmacia Biotech). The ELISA (Genzyme) was calibrated with recombinant human Epo.

Bioassay—The bioactivity of samples (in IU) was determined in vitro essentially as described by Krystal (12). The laboratory standard of recombinant Epo used to generate the standard curve was calibrated against the World Health Organization Second International Reference Preparation. Each sample was diluted with bioassay medium containing 78% α-minimum Eagle’s medium, 20% heat-inactivated fetal bovine serum, 1% β-mercaptoethanol, and 1% penicillin/streptomycin/fungicide (Life Technologies, Inc.).

In Vivo Biological Activity—Three groups of C57BL/6J mice (8–10-week-old females) were used. Before administration of Epo or Epo-Epo, each animal was anesthetized, and its hematocrit was determined using blood obtained by filling two heparinized microhematocrit tubes from the retro-orbital venous plexus. An identifying mark was placed on each animal to permit the determination of its pre- and post-treatment hematocrit individually. The animals were weighed, and each received an identical amount (in IU) of Epo or Epo-Epo fusion protein by subcutaneous injection. The biological activities of the treatment samples were verified in triplicate by in vitro bioassay. The frequency of treatment was once only (day 1). On day 8, the post-treatment hematocrit of each animal was determined. Student’s t test was used to determine whether the mean changes between the pre- and post-treatment hematocrits of each treatment group were different. An α level of 0.05 (two-sided test) was used.

In Vivo Pharmacokinetics—Two groups of female CD-1 mice (8–10 weeks old) were used. Before the administration of Epo or Epo-Epo, 40–50 μl of blood were collected from the tail vein into heparinized microhematocrit tubes. The tubes were centrifuged, and the plasma was collected and frozen at ~80 °C. Each animal received 6 IU of Epo or Epo-Epo by intravenous injection, and blood samples were obtained at 5 min, 1 h, 2 h, 4 h, 8 h, and 24 h thereafter. The injections were performed by a technical specialist staff member of the Beth Israel Deaconess Medical Center Animal Research Facility. The integrity of each injection and the absence of extravasation were confirmed by an independent observer.

RESULTS

Construction and Expression of Epo-Epo cDNA—We constructed an Epo-Epo fusion protein cDNA (Fig. 1) beginning at the 5′ end with the complete coding region of the 193-amino acid human Epo pre-protein (13, 14) with no stop codon (Domain A). This is followed by 51 nucleotides encoding a flexible peptide linker of the sequence A-[G-G-G-G-S]3-T. Downstream of the linker is a domain encoding the 166-amino acid mature Epo protein followed by a stop codon (Domain B). COS1 cells were transfected with either conventional Epo cDNA (8, 12) or Epo-Epo cDNA, and Northern blot analyses were carried out (Fig. 2). Cells transfected with Epo cDNA expressed a 2.0-kilobase transcript. An approximately equal amount of a transcript of ~2.5 kilobases was expressed by cells transfected with Epo-Epo cDNA.

Efficient translation of the fusion protein message and secretion of Epo-Epo by the transfected COS1 cells were demonstrated by SDS-PAGE and Western blot analysis using an anti-Epo monoclonal antibody (10) (Fig. 3). Epo migrated as a broad band with an average apparent molecular mass of 37 kDa, consistent with the glycosylation of the 18.4-kDa polypeptide (15) at each of its four sugar attachment sites. In contrast, Epo-Epo migrated with an average apparent molecular mass of 76 kDa. This is what would be predicted from efficient glycosylation of the four sugar attachment sites in each domain (37 kDa + 37 kDa) with the addition of the 17-amino acid peptide linker sequence (1.8 kDa). This result implies little or no steric hindrance of the co-translational glycosylation and oligosaccharide processing of the fusion protein.

The results of N-deglycosylation support this conclusion. Treatment of Epo-Epo with an optimal concentration of N-glycanase (16) yielded a species that migrated on SDS-PAGE as a narrow band with an average apparent molecular mass of 42 kDa, consistent with the removal of six highly branched complex oligosaccharides (Fig. 4). The use of sub-optimal amounts
of the enzyme resulted in a typical ladder pattern, consistent with stepwise removal of multiple N-linked oligosaccharides. The magnitude of the reduction in average apparent molecular mass (~34 kDa) achieved by complete N-deglycosylation of Epo-Epo is twice that reported for N-deglycosylation of the Epo monomer (15.5–18 kDa) (15, 17, 18). In some experiments, the N-deglycosylated Epo-Epo migrated on SDS-PAGE as a closely spaced doublet, suggesting that some molecules lack the O-linked sugar. This has also been reported for monomeric Epo (15). Taken together, our results are consistent with an Epo-Epo polypeptide backbone to which six N-linked complex oligosaccharides similar in molecular mass to those reported for monomeric Epo are attached. Additionally, as with monomeric Epo, the degree of O-linked glycosylation of Epo-Epo exhibits some variability.

**Table I**

| Construct | Protein secreted | Specific activity |
|-----------|-----------------|-----------------|
|           | Bioassay | ELISA | Bioassay | ELISA |
| Epo<sup>wt</sup> | 3.5–9.2 | 0.010–0.026 | 352 | 13 |
| Epo<sup>wt</sup>/Epo<sup>wt</sup> | 110–195 | 0.109–0.194 | 1007 | 76 |
| Epo<sup>103A</sup>/Epo<sup>wt</sup> | 94–172 | 0.196–0.358 | 480 | 36 |
| Epo<sup>wt</sup>/Epo<sup>103A</sup> | 89–157 | 0.172–0.304 | 516 | 39 |
| Epo<sup>103A</sup>/Epo<sup>103A</sup> | ND | ND |

<sup>a</sup> Epo and Epo-Epo in COS1 cell supernatants were quantified by *in vitro* bioassay and by ELISA. The values are the range of three separate experiments.

<sup>b</sup> Specific activity was calculated by dividing the bioassay value (IU/ml) by the ELISA value (µg/ml).

<sup>c</sup> ND, none detected.

**Biological Activity of Epo-Epo in Vitro**—Epo-Epo is highly biologically active in *in vitro*. We transfected COS1 cells with either conventional Epo<sup>wt</sup> cDNA or Epo-Epo (Epo<sup>wt</sup>/Epo<sup>wt</sup>) cDNA. After 3 days, the supernatants were harvested and subjected to an *in vitro* bioassay. Protein was measured by ELISA calibrated against recombinant human Epo (Table I). Epo<sup>wt</sup> was expressed at modest levels (3.5–9.2 IU/ml; 0.010–0.026 µg/ml) and exhibited a mean specific activity of 352 IU/µg (13 IU/pmol). This specific activity is slightly higher than that obtained by us previously for wild-type Epo expressed in COS1 cells (201–284 IU/µg) (10). In marked contrast, we found a much higher level of expression of Epo<sup>wt</sup>/Epo<sup>wt</sup> (110–195 IU/ml; 0.196–0.358 µg/ml), which exhibited a substantially higher mean specific activity (1007 IU/µg; 76 IU/pmol).

Both Epo domains of Epo<sup>wt</sup>/Epo<sup>wt</sup> are biologically active. We showed this by mutation of arginine 103 (or the arginine 103 equivalent in Domain B, which is arginine 223 of the fusion protein) to alanine (R103A). This mutation results in complete inactivation of the Epo<sup>wt</sup> protein (19, 20). We prepared three different constructs that incorporated this R103A mutation: (a) Epo<sup>R103A</sup>/Epo<sup>wt</sup>, (b) Epo<sup>wt</sup>/Epo<sup>R103A</sup>, and (c) Epo<sup>wt</sup>/Epo<sup>R103A</sup>. We reasoned that if each of the two domains were active, then mutating only one domain would result in a protein that still retained biological activity, but with some decrease in specific activity. Only the double mutant Epo<sup>wt</sup>/Epo<sup>R103A</sup> should be inactive. As seen in Table I, both Epo<sup>R103A</sup>/Epo<sup>wt</sup> and Epo<sup>wt</sup>/Epo<sup>R103A</sup> were expressed efficiently, and both exhibited biological activity. Interestingly, the mean specific activities of the two mutants were 480 (36 IU/pmol) and 516 IU/µg (39 IU/pmol), respectively, essentially one-half that of the non-mutated Epo<sup>wt</sup>/Epo<sup>wt</sup> (1007 IU/µg; 76 IU/pmol). This result strongly suggests that introduction of the R103A mutation into each Epo domain resulted in inactivation of that domain, allowing the other, wild-type domain to activate the receptor.

This indicates that both Epo<sup>wt</sup> domains of Epo<sup>wt</sup>/Epo<sup>wt</sup> are biologically active, i.e. independently capable of receptor activation, and nearly equally so. The double mutant Epo<sup>R103A</sup>/Epo<sup>R103A</sup> was expressed at the mRNA level (data not shown). However, no activity or protein was detected in the COS1 supernatant.

**In Vivo Biological Activity and Pharmacokinetics**—Epo-Epo exhibited enhanced activity in *vivo* compared with monomeric Epo (Fig. 5). We obtained individual pretreatment hematocrits from three groups of four mice. Each animal in the first group received 300 IU Epo-Epo/kg subcutaneously in the form of COS1 cell supernatant, whereas each animal in the second group received a single injection of 300 IU Epo/kg subcutaneously. Supernatant medium from COS1 cells transfected with vector alone was administered to the third group as a control.

Seven days later, the post-treatment hematocrit of each animal
was determined. A substantial increase in hematocrit was observed in all four animals treated with Epo-Epo. In contrast, none of the Epo-treated animals exhibited a significant increase in hematocrit, an expected result in view of the relatively short half-life of Epo. The hematocrit of the Epo-Epo-treated animals increased an average of 2.5% compared with a mean decrease of −0.2% in the Epo-treated group and a mean decrease of −0.8% in the control group. The mean change in hematocrit of the Epo-Epo-treated group was significantly different from that of the Epo-treated group (p = 0.015) and that of the control group (p = 0.008).

We showed that the pharmacokinetic behavior of Epo-Epo is markedly different from that of monomeric Epo (Fig. 6). We injected two groups of mice intravenously either with 6 IU of monomeric Epo or with 6 IU Epo-Epo. At specified times, blood samples were obtained, and the plasma concentration of Epo or Epo-Epo was determined by ELISA. The plasma levels of mice injected with monomeric Epo decreased rapidly (Fig. 6A). In three of the four animals, the Epo concentration decreased to less than half of the peak (5 min) level within 1 h. Epo was detected in the plasma of only one animal after 4 h. In contrast, the plasma levels of all four animals injected with Epo-Epo remained high for many hours after injection (Fig. 6B). Epo-Epo levels remained detectable after 8 h in all four animals and were above 50% of the peak in two of the four animals. Epo-Epo was detectable in the plasma of two of the four animals even after 24 h. Epo-Epo also exhibited another, unexpected pharmacokinetic property. In three of the four animals, Epo-Epo levels continued to rise after the 5-min time point, peaking at 2 h after injection. This unanticipated finding was not due to variations in injection technique. All intravenous injections were performed by skilled technical personnel and monitored independently.

**DISCUSSION**

In the present study, we have shown that a fusion protein consisting of two complete human Epo domains separated by a flexible peptide linker has significantly enhanced *in vitro* and *in vivo* biological activity compared with the monomeric form of recombinant Epo. This enhanced activity appears to be due to an increased specific activity (Table I) coupled with a different pharmacokinetic profile. The unusual geometry of the Epo-Epo pharmacokinetics observed in three of the four animals injected with Epo-Epo (Fig. 6) remains unexplained. We carried out the ELISA analysis of the plasma samples twice at several dilutions and ruled out the possibility of the "high dose hook effect," that is, the paradoxical increase in signal with an increasing dilution of highly concentrated samples (21). The possibility exists that Epo-Epo may associate with itself or with one or more other proteins in the plasma, especially at higher Epo-Epo concentrations. Such an association could interfere with antibody binding in the assay, resulting in a spuriously low reading. The unusual pharmacokinetic behavior of Epo-Epo might also be explained by a rapid and reversible interaction with receptors or other binding sites on cells in close apposition to the plasma, *viz.*, vascular endothelial cells. Both the presence of erythropoietin receptors on these cells and a cellular response to its binding have been documented (22–26).

Previously, we produced chemically linked Epo dimers and showed that we could achieve an increase in potency and a decreased frequency of injection, all leading to enhanced *in vivo* action (6). These chemically linked dimers are, in all likelihood, a mixed population of molecules, presumably consisting of more highly active isoforms and, possibly, less active ones. The use of the fusion protein strategy in the present study provided reasonable assurance of structural homogeneity. Although the construction of the Epo-Epo cDNA was relatively straightforward, there were several uncertainties about the ultimate success of the design that could only be answered through experimentation. Specifically, they related to (a) biological activity, (b) potency, (c) the possibility of intramolecular steric hindrance, (d) post-translational processing, (e) stability, and (f) *in vivo* action.

Based upon our earlier studies, we had concluded that the amino terminus of erythropoietin is not involved in its biological activity because antibodies directed to the first 26 amino acids are not neutralizing (27). However, others have shown that arginine 14 may be important in the action of Epo (19). Therefore, it was unclear whether the second Epo domain (Domain B), that which is tethered by its amino terminus to the...
peptide linker, would exhibit biological activity. An even greater uncertainty existed regarding the first Epo domain (Domain A) with a tethered carboxyl terminus, because Fibi et al. (28) demonstrated that antibodies to the carboxyl terminus of erythropoietin are neutralizing. Furthermore, the proximity of cysteine 161 of Domain A to the linker peptide raised questions regarding the fidelity with which the disulfide bond between cysteine 161 and cysteine 7, which is essential for biological activity, would be formed. However, the data show that both Epo domains are active. Presumably, utilization of the flexible linker sequence similar to that used for an interleukin-3/granulocyte macrophage colony-stimulating factor fusion protein (29) permitted proper folding of both Epo domains into their native conformations.

The recent publication of the crystal structure of Epo bound to two extracellular binding domains of the EpoR (30) shows that the amino acids Ser5, Arg10, Glu13, Leu16, and Leu17 of Epo have minor interactions with EpoR in forming the site 1 intermolecular contact area, whereas Leu5, Asp5, and Arg10 of Epo have minor interactions in forming site 2. In contrast, Val11 and Arg14 of Epo have major interactions at site 2. Near the carboxyl terminus of Epo, residues Asn147, Arg150, and Gly151 have major interactions in forming site 1. These observations would suggest that the length of the peptide linker separating the two Epo domains may be critical in allowing sufficient steric freedom so that each Epo domain of Epowt/Epowt can bind to and induce dimerization of two EpoR.

Curtis et al. (29) constructed a fusion protein (designated PIXY321) consisting of granulocyte macrophage colony-stimulating factor and interleukin-3 linked by an 11-amino acid linker of the sequence (G)12–17S-(G)5–12S (~40 Å) and another fusion protein consisting of interleukin-3 and granulocyte macrophage colony-stimulating factor linked by a 15-amino acid linker of the sequence (G-G-G-G-S)12 (~55 Å). Both of these fusion proteins could bind to cell surface receptors through either cytokine domain, and both exhibited biological activity in vitro consistent with unimpeded function of both cytokine domains. In contrast, a recent study by Qiu et al. (8) used shorter linkers of the sequence (G)12–17S (~10–25 Å) to form fusion proteins of the structure EpoR103A/EpoR103A or Epo wt/ Epo wt. Interestingly, these EpoR103A/EpoR103A fusion proteins were biologically active (although less so than Epo wt), apparently because the site 1 intermolecular contact area of each EpoR103A domain bound one EpoR, and because the linkers were short and flexible enough to allow receptor dimerization by the (theoretically) inactive fusion protein. However, in contrast to our results, Qiu et al. (8) observed no increase but rather a moderate decrease in the activity of their Epo wt/Epo wt fusion proteins compared with Epo wt. Taking into consideration the results of Curtis et al. (29) and the results of the present study, along with the structural data (23), the short linkers used by Qiu et al. (8) probably allowed each Epo wt domain of their Epo wt/Epo wt fusion proteins to bind just one EpoR, resulting in an activity similar to that observed by them for Epo wt and for their EpoR103A/EpoR103A. Apparently, the 17-amino acid linker (A-[G-G-G-G-S]12T) (~63 Å) used in this study of Epo-Epo allowed both Epo domains to function unimpeded and resulted in enhanced activity.

The potency of our Epo-Epo in vitro is significantly higher than that of conventional recombinant Epo. It is 3-fold higher per microgram and 6-fold higher on a per mole basis. There are several possible explanations for this observation. It may be due to an increased stability of Epo-Epo or to a difference in endocytotic efficiency. Alternatively, this increase in potency may reflect an increase in receptor affinity. Although speculative, the possibility exists that the binding of one domain of Epo-Epo facilitates the binding of the second domain, i.e. positive cooperativity. In this regard, we have shown previously that the induction of EpoR clustering by dimethyl sulfoxide treatment of Rauscher murine erythroleukemia cells resulted in positive cooperativity and an enhanced biological response (31, 32). The sequential or simultaneous binding of both Epo domains of the fusion protein might also induce such receptor clusters, thereby leading to increased local concentrations of signal transduction mediators that could result in enhanced signaling.

It is notable that the secretion of EpoR103A/Epo wt in this study was 8-fold that of Epo wt (Table I). However, the transcript levels seen on Northern blots were approximately equal (Fig. 2), indicating that an increase in transcription was not responsible. Other potential causes for the difference in secretion include more efficient translation, an increased intracellular trafficking, and enhanced stability during biosynthesis and secretion. Interestingly, both EpoR103A/Epo wt and Epo wt/ EpoR103A were secreted at levels greater than that of Epo wt/ Epo wt itself. This finding supports the hypothesis that protein stability may play a role in the enhanced secretion of the fusion protein. Our previous work showed that mutations at arginine 103 of Epo could lead to a molecule with increased stability (10, 33). Thus, because single R103A mutations in the fusion protein (which presumably enhance its stability) lead to increased rates of secretion, it seems plausible that the enhanced stability of Epo wt/Epo wt over Epo wt plays a role in the higher levels of secretion of the fusion protein.

Although our EpoR103A/EpoR103A mutant was expressed at the mRNA level, we detected no protein by either bioassay or ELISA. This result is in contrast to that reported by Qiu et al. (8) for their dimeric R103A fusion proteins with relatively short Gly3, Gly5, or Gly7 linkers that were secreted efficiently by COS7 cells. Although speculative, we believe that these differing results suggest that the length and composition of the linker may play a role. Substitutions at R103 affect the activity and thermal stability of the monomer. They may also influence potential interactions between and among the Epo domains of the fusion protein and the linker itself, thereby altering the protein’s interaction with chaperons and its folding and secretion efficiency.

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