Homodimerization Restores Biological Activity to an Inactive Erythropoietin Mutant*

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Erythropoietin (Epo) is believed to transduce a signal by bringing two Epo receptors into close proximity, enabling cross-phosphorylation. We compared monomeric Epos with homodimers in which two Epo monomers are linked by polyglycine. Monomeric Epo mutant R103A is unable to support Epo-dependent cell growth or trigger Janus kinase 2 and STAT5 activation, even at concentrations greater than 7,000 times that sufficient for wild-type Epo activity. In contrast, R103A homodimer induces proliferation and transduces signal at concentrations similar to that of wild-type Epo monomer and homodimer. These experiments show that two discrete domains on Epo are required for receptor binding and activation. Our results also suggest that the EpoR can be dimerized by different forms and sizes of molecules, as long as two recognition motifs are provided in the same molecule. Design of other dimeric molecules may enhance our understanding of cytokine specificity and signal transduction.

The biological activity of a number of cytokines depends upon ligand-induced aggregation of their cognate receptors, as exemplified most convincingly by structural and biochemical studies of human growth hormone and its receptor (1–5). A similar mechanism has been proposed for receptor activation by erythropoietin (Epo),† the primary regulator of red blood cell production (6–9). The binding of Epo to its receptor (EpoR) initiates a complex signal transduction cascade that includes the JAK-STAT, phosphatidylinositol-3 kinase, and Ras pathways (10, 11). Epo-specific signal transduction can be induced in the absence of ligand by a mutant EpoR in which a cysteine replacement in the extracellular domain enables two receptor molecules to form a disulfide bond (12). Moreover, a synthetic peptide bearing no resemblance to Epo can also transduce a signal by binding to two EpoR molecules (13, 14).

These observations raise the question of how a nonsymmetrical protein monomer specifically activates its cognate receptor on the cell surface. There is no direct information to date on Epo’s three-dimensional structure. Expression and characterization of a large number of site-directed Epo mutants support a four α-helical bundle structural model (15), resembling that of other cytokines (16). Several of these Epo mutants have been shown to be deficient or lacking in biological activity (17–20). These structure-function studies have led to the proposal of two domains on Epo that bind to its receptor: one at Arg19, Gly151, Lys152, and the other at Arg103, Arg105, and possibly Ser104 (18–20). Mutants that nullify either of the two domains would be unable to bind to two EpoR molecules. Among the mutants tested to date, R103A has the lowest specific bioactivity (17–19). No proliferation activity was detectable even at high concentrations. Despite this, R103A Epo is able to bind tightly to its receptor (19) and therefore is likely to retain one high affinity domain. We designed dimeric forms of Epo in which two R103A Epo mutants were tandemly attached by a flexible isopeptide linkage and asked whether the presence of a second postulated high affinity binding motif in the same molecule could resurrect R103A’s activity. We demonstrate that the Arg103 homodimers were biologically active, stimulating proliferation of human leukemia cell line UT7/Epo, and transducing Epo-specific signaling. These results provide direct evidence for the two-domain model of erythropoietin function.

EXPERIMENTAL PROCEDURES

Plasmids—The construction of plasmid vectors expressing monomeric Epo was reported previously (15, 18). Briefly, a 943-base pair EcoRI-BglII fragment, which includes the complete coding sequence of the wild-type human erythropoietin as well as untranslated regions 216 base pairs upstream and 183 base pairs downstream, was inserted into the mammalian expression plasmid pSG5 (Stratagene) and designated pSG5-EPO/WT. To enhance Epo purification, a His tag was engineered immediately adjacent to the C terminus by means of PCR with oligonucleotide primers EcoRT (5′–GGC GAA TTC CCC GGA GCC G–3′) and BamHisB (5′–TCA CCC GGA TCC TCA GTG GTG GTG GTG TTC GTC GCC TGG CCT GGT GGC G–3′). The amplification products were purified and inserted into the EcoRI and BamHI site of pSG5, and the resulting vector was designated pSG5-EPO.His/WT. pSG5-EPO.His/R103A was made by the same pair of PCR primers using pSG5-EPO/WT as template.

Plasmids containing dimeric Epo DNA were made from a three-way ligation of two PCR-amplified monomeric units into EcoRI and BamHI sites of parental plasmid pSG5. pSG5-Epo/WT and pSG5-Epo/R103A DNA were used as templates. The primers used were: EcoRT (see above) and BsiEB (5′–GAA GTG CCG TCG CCT GTC CAG GGG CCG TC–3′) for the first monomer and BsiEI/Gly3:5′–GAA GTG CCG AGG CCG GGG CGC CCC ACC ACG CC–3′; the primer sequences for 5′, 7, and 9 Gly were similar to the Gly3 primer and thus not shown) and BamHB (5′–TCA CCC GGA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TTC GTC GCC TGG CCT CTT GCA GGC G–3′) for the second monomer. The two PCR products were digested with EcoRI/BsiEI and BsiEI/BamHI, respectively, and ligated together with EcoRI/BamHI digested pSG5 vector. The His-tagged Epo DNA was prepared by PCR in the same way except that BamHB was replaced with BamHisB primer (see above). In the preparation of the R103A dimers, BamI-Arg/BisB(5′–TCA CCC GGA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TCA TTC GTC GCC TGG CCT CTT GCA GGC G–3′) was used to make a polyhistidine tag adjacent to Arg105 of the second monomer. This was done to avoid the suspected cleavage of the poly-His tag at Arg105 which is normally the last amino acid of...
Epo immediately N-terminal to the engineered His tag. The Ala mutation in both monomeric units of R103A dimer was confirmed by dideoxy sequencing of BstE-digested and purified monomeric DNA fragments.

Production of Monomeric and Dimeric Epo in Mammalian Cells—COS7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 15% fetal bovine serum and 1% penicillin/streptomycin. For transient expression of Epo, COS7 cells were grown to 60–80% confluency, transfected with 20 μg of recombinant plasmid DNA per 10-cm dish using the calcium phosphate precipitation protocol. 20 μg plasmid pADVantage (Promega) were co-transfected to enhance the yield of protein production. 72 h after transfection, the medium was filtered and subjected to radiomunoassay (RIA) to determine secreted Epo concentration. For the transfection of plasmids containing His6-tagged Epo, spent medium was removed 24 h after transfection, and COS7 cells were allowed to grow for 2 more days in serum-free medium before collecting the supernatant. Medium containing His6-tagged proteins was loaded onto a Ni-nitrilotriacetic acid metal Chelex column (Qiagen) for affinity purification.

Bioassays—The dose-dependent proliferation activities of wt Epo and Epo mutants were assayed in vitro using an Epo-responsive target cell UT7/Epo, a human cell line derived from the bone marrow of a patient with acute megakaryoblastic leukemia (22). Briefly, cells were washed with PBS and mixed with various amounts of Epo in a 96-well plate, with approximately 104 cells/well; after 72 h of incubation, cellular growth was determined by [3H]thymidine (NEN Life Science Products) incorporation (23) and colorimetric proliferation assay (Promega).

Immunoprecipitation and Western Blot—1 × 107 UT7/Epo cells were starved in medium lacking Epo for 16 h and then treated with various forms of Epo for 5 min. Cells were lysed in 0.5 ml of lysis buffer containing 1% Triton X-100 and 100 μM sodium vanadate, and immunoprecipitated with antibody against JAK2 (Upstate Biotechnology). Samples were electrophoresed on a 7% SDS-polyacrylamide gel and Western blotted with an antibody against phosphotyrosine (4G10, Upstate Biotechnology) using enhanced chemical luminescence (Promega).

Electrophoretic Mobility Shift Assay—UT7/Epo cells were starved overnight and stimulated with Epo for 5 min. Cells were washed, collected, and used for preparation of nuclear extracts. Nuclear extracts (5 μg) were incubated with 0.25 ng of 32P-labeled STAT5 consensus binding sequence (5’-AGA TTT CTA GGA ATT CAA TCC-3’; Santa Cruz Biotechnology) for 20 min at room temperature and electrophoresed on a 5% polyacrylamide gel in 0.5× TBE buffer. In the supershift experiment, 1 μg of anti-STAT5 (Santa Cruz Biotechnology) antibody was added following the initial incubation and reincubated at room temperature for another 15 min before electrophoresis.

RESULTS

Expression of Dimeric Epo in COS7 Cells—The R103A dimers and WT dimers were constructed by PCR (Fig. 1) and prepared by transient expression and secretion in mammalian COS7 cells. Concentration of Epo products was determined by a RIA using a polyclonal antiserum against Epo (15, 21, 22). Studies employing conformation-dependent monoclonal antibodies indicate that R103A is properly folded into native tertiary structure (24, 25) and therefore would be expected to bind as avidly as WT Epo to the polyclonal antiserum. We found that dimeric Epos were secreted from COS7 cells at levels comparable to that of monomeric Epo (in the range of 150–500 units/ml). The fact that these dimers are recognized by Epo antisera in the RIA suggests that their tertiary structures are intact.

Epo-dependent Cell Proliferation—The biological activity of R103A dimer was assessed in two ways: 1) in vitro proliferation in UT7/Epo cells, an Epo-responsive human cell line (22); and 2) activation of Epo-specific signal transduction monitored by Jak2 phosphorylation and activation of STAT5 binding to DNA.

The proliferation bioassay shown in Fig. 2 compares the dose response curves of UT7/Epo cells exposed to WT Epo and the R103A mutant, both as monomers and as homodimers. Proliferation generally requires >50 milliunits/ml monomeric WT Epo (EC₅₀ = 105 milliunits/ml). A similar biological effect was observed at the same concentrations of dimeric WT Epo (EC₅₀ = 185 milliunits/ml). As shown previously (17–19), monomeric R103A was completely inert even at a concentration of 350,000 milliunits/ml, at least 7,000 times that sufficient for WT Epo activity (Fig. 2). In contrast, dimeric R103A was biologically active at ~150 milliunits/ml (EC₅₀ ~ 400 milliunits/ml), slightly higher than that required for dimeric WT Epo. The biological activities of the dimeric WT Epo and dimeric R103A were not affected by the number (3–7) of glycine spacers.

JAK2 Phosphorylation and STAT5 Activation upon Epo Stimulation—Analysis of Epo-dependent signal transduction, as shown in Fig. 3, A and B, was in full agreement with the cell proliferation results described above. Phosphorylation of JAK2, a member of the Janus kinase family, is thought to be the first step of Epo-specific signal transduction upon EpoR activation. As expected from previous studies (26), the addition of WT Epo to UT7/Epo cells, in doses as low as 0.5 unit/ml, resulted in...
prompt tyrosine phosphorylation of JAK2. In contrast, addition of up to a 640-fold higher dose of monomeric R103A failed to induce JAK2 phosphorylation (Fig. 3A). However, in keeping with the bioassay results in Fig. 2A, dimers of both WT Epo and R103A, when added to UT7/Epo cells, elicited robust phosphorylation of JAK2 (Fig. 3B).

Several signal transduction pathways subsequent to JAK2 phosphorylation have been shown to be activated by Epo (10, 11). One which has been thoroughly studied is the JAK-STAT pathway in which STAT5 protein is recruited by activated EpoR and becomes phosphorylated by JAK2. Phosphorylated and dimerized STAT5 protein is translocated into the nucleus where it binds to consensus DNA sequences and transactivates gene expression. To test whether the Epo dimers utilize the same pathways, the binding of activated STAT5 protein to its consensus promoter sequence was analyzed by electrophoretic mobility shift assay, as shown in Fig. 4. R103A dimers induced STAT5 activation, suggesting that, like wild-type Epo, they triggered signaling along the JAK-STAT pathway.

Immunoblot of the Engineered Tag for Confirmation of Protein Concentration—To rule out a significant error in our measurement of Epo concentration by immunoassay, we devised an independent way of measuring the absolute concentration of our recombinant Epo products. The coding regions were extended to include six histidine residues at the C terminus of monomeric and dimeric Epo (attached to Asp¹⁶⁵ or Arg¹⁶⁶ of the second monomer unit) (Fig. 1). The C-terminal poly-His tag does not affect Epo’s specific bioactivity (15) (data not shown). WT and R103A Epo monomer and dimer samples estimated by RIA to contain the same amounts of Epo were run on a denaturing SDS-PAGE gel, and blotted with monoclonal antibodies against the poly-His tag (Fig. 5). For Epo monomers, the density scan of the blot agreed well with the RIA units (within a factor of two), consistent with a previous study which demonstrated by use of conformation-sensitive antibodies that the Arg¹⁰³ → Ala mutation has no apparent effect on the overall folding of Epo (20, 25). The RIA underestimated the concentration of WT dimers by a factor of 2–5. This may explain why the WT dimer appeared to be slightly more active than Arg¹⁰³ dimmer in our bioassay (Fig. 2). Poly-His-tagged R103A dimer had approximately the same Western blot signal as that of the two monomers (Fig. 5). Thus our estimate of the specific bioactivity of dimeric R103A is not confounded by use of excessive protein, and can be confidently attributed to the presence of the second monomer.

DISCUSSION

The simplest explanation of the results in Figs. 2–4 is that the dimeric R103A Epo has two active receptor binding domains, each from one R103A monomeric unit. They each bind to one EpoR molecule, and bring them into close enough proximity to enable activation. Thus the deficient receptor binding domain in R103A can be compensated by the active binding site of a second R103A Epo which is covalently linked in the dimer construction.

In principle, parallel experiments could be performed with monomeric and dimeric mutants at positions 150–152, the sites on helix D that have been proposed to comprise an independent receptor-binding domain. However, in contrast to

FIG. 3. Tyrosine phosphorylation of Janus kinase 2 (JAK2) protein upon Epo monomer (panel A) and dimer (panel B) stimulation. A, UT7/Epo cells were starved overnight and collected 5 min after addition of WT or mutant (R103A) monomeric Epo. The cell lysate were subjected to immunoprecipitation with JAK2 antibody and Western blotted with antibody against phosphotyrosine (top) and JAK2 (bottom). B, comparison of JAK2 phosphorylation stimulated by WT and R103A Epo monomers and dimers.

FIG. 4. Electrophoretic mobility shift assay of nuclear extracts made from UT7/Epo cells treated with WT or R103A monomeric and dimeric Epos. 30 units/ml Epo was used unless otherwise specified. ³²P-Labeled STAT5 consensus sequence was used as the probe. Migration of STAT5 protein-DNA complex is marked by a solid arrow. Lane 1, buffer control; lane 2, UT7/Epo nuclear extract with no Epo treatment; lane 3, WT monomer; lanes 4 and 5, R103A monomer, 30 and 300 units/ml, respectively; lane 6, WT dimer; lanes 7–9, R103A dimer with 3, 5, and 7 Gly linkers. Lanes 10–18 are the same as lanes 1–9 except that an antibody against STAT5 proteins was added to super-shift the protein-DNA complex (marked by dashed arrow).

FIG. 5. Comparison of monomer and dimer Epo concentration using Western blot. Epo monomer and dimer samples estimated by RIA to contain the same amounts of Epo (2.5, 5, and 10 units of each species) were run on a denaturing SDS-PAGE gel and blotted with monoclonal antibodies against the poly-His tag.
R103A which is completely "dead," the mutants on helix D that have been studied so far (18–20) show either impaired folding into native tertiary structure or only partial diminution of specific biological activity. Therefore, data obtained from homodimers of these mutants would be difficult to interpret.

Interestingly, the wild-type monomeric Epo is significantly more active than the wild-type dimer and R103A dimer. Although more sophisticated quantitation is required to address this question, one possible explanation is that the dimeric Epo molecules impose steric hindrance when binding to receptor, resulting in a lower binding affinity. The dose dependence curves of R103A dimer (Fig. 2) are slightly steeper than those of both monomeric and dimeric Epo, suggesting possible cooperativity. This is consistent with the current model of Epo function (19) in which the R103A dimer would be composed of two tight binding sites, and thus recruitment of the second receptor would be more efficient than with the proposed weak secondary binding site in wild-type Epo.

Our experiments comparing monomeric and dimeric R103A clearly indicate that two domains (motifs) on the Epo molecule are required for its biological function and strongly support the binding of a single molecule of Epo to two receptor molecules. As shown in Fig. 6, two receptors can be brought into juxtaposition for activation by five different mechanisms: (a) a single native Epo molecule containing two different and independent receptor binding motifs (18–20); (b) as shown in this study, a dimeric Epo, containing only one binding motif on each monomer; (c) a mutant EpoR which is cross-linked via a disulfide bond; (d) a bifunctional antibody against the extracellular portion of the Epo receptor (8, 9); and (e) an EpoR-binding mimetic peptide (13, 14). The length of linker in the dimeric Epo does not seem to be important (Figs. 2 and 3). Mutant dimers having spacers of 3, 5, or 7 glycine residues all gave similar results. A ligand can be effective as long as it has two recognition/binding motifs in the same monomeric molecule, or one motif in each subunit of a dimer.

The mimetic peptide binds to two EpoR molecules as a homodimer; thus the complex has 2–2 stoichiometry and almost perfect 2-fold symmetry (13, 14). It follows that the Epo-Epo dimer complex reported here should also have 2-fold symmetry. However, native Epo monomers are nonsymmetrical, and Epo’s two receptor binding sites appear to have different affinities for EpoR (19). It will be of interest to see whether the nonsymmetrical Epo molecule interacts with other accessory proteins. Additional Epo receptor subunits have been proposed (27–30), but not yet characterized. In the absence of high resolution structure of the Epo-EpoR complex, there is the formal possibility that the two domains of Epo have different functions. For example the domain that includes Arg103 may bind to another Epo molecule or to an accessory protein, and thus indirectly interacts with the Epo receptor.

The design and evaluation of peptide-linked homodimers may be applied to other cytokines or growth factors whose signaling function depends on bringing receptor subunits into apposition. Synthetic molecules (for bioavailability) or large protein molecules (for enhanced activity or stability) can be evaluated as replacements for the native proteins currently in clinical use.

REFERENCES