Mutagenesis Studies of the Human Erythropoietin Receptor

ESTABLISHMENT OF STRUCTURE-FUNCTION RELATIONSHIPS*  

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Mutagenesis of the erythropoietin receptor (EPOR) permits analysis of the contribution that individual amino acid residues make to erythropoietin (EPO) binding. We employed both random and site-specific mutagenesis to determine the function of amino acid residues in the extracellular domain (referred to as EPO binding protein, EBP) of the EPOR. Residues were chosen for site-specific alanine substitution based on the results of the random mutagenesis or on their homology to residues that are conserved or have been reported to be involved in ligand binding in other receptors of the cytokine receptor family. Site-specific mutants were expressed in *Escherichia coli* as soluble EBP and analyzed for EPO binding in several different assay formats. In addition, selected mutant proteins were expressed as full-length EPOR on the surface of COS cells and analyzed for 125I-EPO binding in receptor binding assays. Using these methods, we have identified residues that appear to be involved in EPO binding as well as other residues, most of which are conserved in receptors of the cytokine receptor family, that appear to be necessary for the proper folding and/or stability of the EPOR. We present correlations between these mutagenesis data and the recently solved crystal structure of the EBP with a peptide ligand.

Erythropoietin (EPO) is a glycoprotein hormone that functions as the primary regulator of erythropoiesis by binding a specific receptor (EPOR) on the surface of erythrocyte precursor cells, signaling their proliferation and differentiation into mature red blood cells (reviewed in Ref. 1). The human EPOR is a 484-amino acid glycoprotein comprised of extracellular and cytoplasmic domains of nearly equal size and a single transmembrane domain (2). The extracellular domain of the EPOR contains a 225-amino acid region referred to as the cytokine receptor homology (CRH) domain that shares conserved features with an expanding family of cytokine and growth factor receptors (3, 4) including the receptors for many interleukins (IL), colony stimulating factors, growth hormone (GH), thrombopoietin, leptin, interferons (IFN), and tissue factor among others. The CRH domains consist of two motifs of approximately 100 amino acids each that are structurally related to fibronectin type III domains. Based on this homology, Bazan (3, 4) proposed that the CRH domains consist of two motifs of seven β-strands each which adopt β-sheet structures with fibronectin type III-like or immunoglobulin (Ig)-like folds. These structural predictions have been confirmed by the solution of the crystal structures of the ligand-bound extracellular domains of the GH receptor and IFN-γ receptor α (IFN-γRα), the GH bound extracellular domain of the prolactin receptor, the extracellular domain of tissue factor, and most recently, the extracellular domain of the EPOR with a peptide ligand (5–10). Alignment of the CRH domains of this family of receptors based on their predicted β-strand secondary structural elements reveals several conserved characteristics (3, 4, 11). Of these, the most highly conserved are four spatially conserved cysteine residues that form two cysteine bridges in the amino-terminal domain and a WSXWS sequence at the membrane proximal end of the carboxyl-terminal domain. The overall amino acid identity between CRH domains in receptors of this family is generally less than 25% (11). Thus, the general structural topography of these domains is more highly conserved than the primary amino acid sequences.

Amino acid residues involved in ligand binding have been identified in several receptors of the cytokine receptor family using alanine substitution site-specific mutagenesis. The importance of many of these ligand binding determinants was subsequently confirmed in structural studies. For example, Woodcock et al. (12) used alanine substitution mutagenesis on the common β-chain (βc) of the human GM-CSF, IL-3, and IL-5 receptors to identify amino acid residues critical for the formation of the high affinity receptors for GM-CSF and IL-5. Using mutation complementation, they also identified a residue in GM-CSF that is likely to interact with the critical residues on the βc. Support for these results was provided by a molecular model of the GM-CSF receptor complex (ligand, α-chain, and βc) in which the binding determinants identified by mutagenesis were predicted to be within bonding distance of each other (13). Alanine substitution mutagenesis has also been used to identify important ligand binding determinants in the extracellular domain of the growth hormone receptor (14), and many
of these residues were subsequently shown to interact with residues on GH in the crystal structure of the GH-growth hormone receptor complex (5). Although the structure of the EBP with a peptide ligand has recently been determined (10), the precise nature of the EPO-EBP interaction remains to be determined. Site-specific mutagenesis provides a sensitive method for investigating which residues of the extracellular domain of the EPOR are involved in EPO binding. In retrospect, we are able to compare the EBP mutagenesis data to the EBP-peptide structure which may help identify amino acid residues that are involved in EPO binding.

Previously, we reported that a single residue, Phe93, is a critical ligand binding determinant of the EPOR (15). Here, in a more extensive mutagenesis analysis of the 225-amino acid extracellular domain of the EPOR, we report the effect on EPO binding of single amino acid substitutions created by site-specific mutagenesis. In some cases, residues were selected for site-specific mutagenesis based on random mutagenesis results. The functions of other amino acid residues were investigated based on their conservation among receptors of the cytokine receptor family or their homology to ligand binding determinants reported for other receptors of this family. Wild-type and mutant proteins were expressed in *Escherichia coli* as soluble EPO binding proteins (EBP), consisting of amino acids 1–225 of the extracellular domain of the mature EPOR (16), and analyzed for EPO binding in several different assay formats. Some mutant proteins were expressed as full-length receptor on the surface of COS cells and analyzed in receptor binding assays with 125I-EPO. We have identified residues that appear to be involved in EPO binding as well as other residues, most of which are conserved in receptors of the cytokine receptor family, that appear to be necessary for the proper folding and/or stability of the EPOR. From these results, we can begin to build the structure-activity relationships that will help to delineate the EPO binding face of the EPOR.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Characterization**—The cloning, expression, protein recovery, and refolding of the EBP has been described in detail elsewhere (16). Briefly, the nucleotide sequence of the first 225 amino acids of the extracellular domain of the human EPOR (EBP) was cloned into plasmid pBSAM3 that contains a synthetic peb signal sequence. Expression of the EBP was under the control of the T7 promoter in *E. coli* strain BL21(DE3)pLysS and was induced by the addition of isopropyl-β-D-thiogalactopyranoside for 3–5 h at 37 °C. The EBP protein was produced as insoluble protein localized to inclusion bodies. The EBP was recovered, solubilized, and refolded as described (16). The protein refolding process was monitored by high performance-size exclusion liquid chromatography (HP-SEC), and the active protein was detected by peak shift analysis through the addition of purified recombinant EPO.

Mutant EBPs created by site-specific mutagenesis were expressed in *E. coli* and purified as described for the wild-type EBP (16). Some mutant EBPs were purified by a modification of these methods in which the hydrophobic interaction chromatography step was replaced by a preparative high-performance-size exclusion chromatography (HP-SEC) step. The purity of mutant and wild-type EBP was estimated using the experimentally determined extinction coefficient for the wild-type EBP of 2.3 absorbance units per mg/ml at 280 nm (16).

**EBP-bead Binding Assay**—EBP mutants were assayed in the EBP-bead binding assay described previously (16). Briefly, 50 μl of EBP beads (wild-type EBP covalently attached to agarose beads) were added to tubes containing varying amounts of 125I-EPU (DuPont NEN, approximately 100 μCi/μg). The reaction volume was increased to 0.5 ml with phosphate-buffered saline, 0.2% BSA, and the beads were rocked gently at room temperature overnight. The reaction mixture was loaded onto a 1.0-ml micro column (Iscolab, and the trapped beads were washed 3 × with 1-ml washes of phosphate-buffered saline, 5.0% BSA. The columns, containing the EBP-bound 125I-EPU, were counted in a gamma counter (ICN Micromed, Huntville, AL).

**Mutagenesis**—Random mutations were introduced into the EBP using the method of Cadwell and Joyce (17). The EBP DNA was amplified by the polymerase chain reaction (PCR) under conditions that would introduce the correct base substitutions by *Taq* polymerase. EBP-specific PCR primers contained a *Sal*I restriction site on the 5′ primer and a *Spe*I restriction site engineered within the 3′ primer. Plasmid pCOMB3 DNA (gift of C. Barbas, Scripps Research Institute) was cut with XhoI and SpeI. Due to an internal XhoI site in the EBP, the EBP-specific PCR primers contained a *Sal*I restriction site which is compatible with XhoI. The PCR amplification conditions were as follows: 10 mm Tris-HCl, pH 8.3, 50 mm KCl, 7 mm MgCl₂, 0.5 mm MnCl₂, 0.2 mm dGTP, 0.2 mm dATP, 1 mm dCTP, 1 mm dTTP, plus 5 units of *Taq* polymerase per 100-μl reaction. Reaction cycle conditions were 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, for 25 cycles on a thermal cycler (Perkin-Elmer 9600). The resulting amplification product was cut with restriction enzymes and ligated into the vector pCOMB3 generating a fusion with the carboxyl-terminal half of the M13 geneII protein (18). The DNA was then transformed into *E. coli* strain XL-1 blue (Stratagene, La Jolla, CA).

In most cases site-specific mutants were generated using the polymerase chain reaction (PCR) in which single primers adjacent to convenient restriction endonuclease sites were designed to include the specific desired mutation. Other mutants required the use of overlap PCR (19) to facilitate site-specific mutagenesis. All mutants were verified by DNA sequence analysis to confirm the presence of the desired mutation and the absence of any unintentional mutations.

**Colony Ligand Blot**—Bacterial colonies containing the random EBP library were tested for 125I-EPO binding using a colony ligand blot assay. Transformed bacterial colonies were picked and grown on LB-amp plates at 37 °C for 4 h. The plates were overlaid with nitrocellulose filters soaked in 5 ml isopropl-β-D-thiogalactopyranoside and placed at 30 °C overnight. The filters were removed from the plates and placed in a chloroform chamber for 15 min and subsequently incubated in lysozyme buffer (50 mm Tris-HCl, pH 8.0, 150 mm NaCl, 5 mm MgCl₂, 3% BSA, 0.4 mg/ml lysozyme, 1 unit/ml DNase) for 1 h with shaking. The filters were incubated in 0.2 mm Tris-HCl, pH 8.0, 5 mm ureda for 45 min, followed by 45 min in 50 mm Tris-HCl, pH 8.0, 0.1 mm ureda. The filters were blocked for 1 h in 5% non-fat milk, 3% BSA in Tris-buffered saline (TBS). Fresh, milk/BSA/TBS containing 125I-EPO (200–300 pM) was added, and the filters were incubated for 2 h at room temperature with shaking. The filters were washed 2 × for 5 min with milk/BSA/ TBS, 2 × for 5 min with TBS, and then dried and exposed to x-ray film (Kodak XAR-5). Colonies were selected non-binding clones on doxyl-endorsement by DNA screening. Colonies were cultured on LB-amp plates at 37 °C for 4 h. The plates were overlaid with nitrocellulose filters soaked in lysozyme buffer, induced for 3 h with 1 mg isopropl-β-D-thiogalactopyranoside, and analyzed by Western blot using anti-EBP antiserum. Selected non-binding clones confirmed to contain the EBP insert and produce a fusion protein of the expected size were grown for DNA purification and sequence analysis.

**Cos Cell Transfection**—HEK-293 cells were maintained in DMEM (Life Technologies, Inc.), 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT), 1% l-glutamine, 1% penicillin, 0.1% streptomycin. Cells were seeded into 100-mm tissue culture dishes at approximately 1.0 × 10⁶ cells per plate. The cells were transfected with the EPOR-containing plasmid (wt or mutant) using 10 μg of DNA/plate and DEAE-dextran (0.14 mg/ml) in DMEM minus fetal calf serum. The cells were incubated overnight at 37 °C with the DNA mixture. Cells were then washed with DMEM-FCS and incubated in DMEM-FCS containing 100 μg chloroquine for 2.5 h at 37 °C. The cells were washed with DMEM-FCS; fresh media were added, and the cells were incubated for 48 h prior to use in the EPO binding assay.

**COS Cell Binding Assay**—Transfected COS cells were removed from the plates with dissociation buffer (phosphate-buffered saline, 0.5 mM EDTA). Cells from duplicate plates were pooled, centrifuged, washed, and resuspended in binding buffer (RPMI 1640, 5% BSA, 25 mM Hepes, pH 7.5, 0.02% sodium azide) and counted. Binding assays were essentially as described (20). Briefly, tubes containing cells and varying concentrations of 125I-labeled EPO were incubated overnight at 4 °C. Nonspecific binding was determined by the addition of at least 100-fold excess of unlabeled EPO. Following incubation, the tubes were centrifuged at 12,000 rpm for 1 min at 4 °C. The supernatant was removed; the cell pellet was resuspended in 100 μl of binding buffer, and the cell suspension was layered onto 0.7 ml of bovine calf serum. The tubes were then centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was removed, and the bottom of the tubes were snipped off and counted in a gamma counter. The binding data were transformed using the method
of Scatchard (21) in order to determine binding affinities. Detection of EPOR on the surface of COS cells was performed using an anti-EBP monoclonal antibody in a modification of the receptor binding assay described above. This procedure for cell surface detection has been described previously (15).

Inhibition of Proliferation Assay—FDC-P1 cells, stably transfected with the human EPO receptor lacking the terminal 40 amino acids (FDC-P1tHER) and demonstrating EPO responsiveness, were used for inhibition of proliferation assays. Briefly, cells were maintained in RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin, and 1.0 unit/ml recombinant human EPO. Cells were deprived of factor for 18 h and subsequently added to 96-well tissue culture plates at a density of $4 \times 10^4$ cells/well. Each well contained cells, 0.3 units/ml EPO, and the wt EBP or EBP mutants at various concentrations. Plates were incubated at 37°C for approximately 42 h, after which the cells were pulsed with 1.0 $\mu$Ci/well of $[3H]$thymidine (20 Ci/mmol, DuPont NEN) for 6 h at 37°C. Cells were harvested onto glass fiber filters (GF/A) using a cell harvester (Tomtec), and the filters were counted in a scintillation counter (LKB Betaplate 1205).

RESULTS

Mutagenesis Strategy—Previously, the EBP was expressed in E. coli, purified, and extensively characterized (16). The EBP is capable of binding EPO with low nM affinity and can be purified in mg quantities from laboratory scale fermentations, making it suitable for mutagenesis studies directed toward the elucidation of the EPO binding face of the EPOR. EBPs were chosen for site-specific mutagenesis based on results of a random mutagenesis study or on their homologous locations to conserved and non-conserved residues reported to be involved in ligand binding in other receptors of the cytokine receptor family. Generally, our approach was to screen for mutations resulting in decreased EPO binding activity by testing crude preparations of the mutant EBPs in a high performance-size exclusion chromatography (HP-SEC) assay (16). Although not quantitative, the HP-SEC assay does not require purification of the mutant proteins, is quick and easy to perform, and is capable of identifying mutations resulting in large reductions in EPO binding (15, 16). This assay was successful in identifying an EBP mutant with a 1,000-fold increased IC$_{50}$ for EPO (15); however, it may not be sensitive enough to detect less dramatic effects on EPO binding, since an EBP mutant (L96A-EBP) with a 200-fold increased IC$_{50}$ for EPO still showed EPO binding. Following HP-SEC analysis, selected mutant proteins were purified and analyzed in competition binding assays or expressed and characterized as full-length receptor on the surface of COS cells. Using this strategy, we have created over 40 single alanine substitution mutants in the extracellular domain of the EPOR. Most of these mutants were initially analyzed for EPO binding activity in the HP-SEC assay, and approximately half were purified and further characterized in the EBP-bead and/or inhibition of proliferation assays.

Site-Specific Mutagenesis of Amino Acids in the EBP—To identify amino acid residues of the EBP involved in EPO binding, random mutagenesis was performed using a PCR mutagenesis method (17). Random mutants were evaluated for the ability to bind $^{125}$I-EPO in a colony ligand blot assay, and the expression of mutants that did not bind EPO was confirmed by Western blot analysis of cell lysates using anti-EBP antiserum. For random mutants exhibiting EBP expression but no EPO binding, DNA encoding the EBP was sequenced to determine the location(s) of the mutation(s). Two mutations (V196D and A198V), resulting in the loss of EPO binding as evaluated by colony ligand blot analysis, involved residues located in the F$^\beta$ strand, boxed region of the F$^\beta$ strand, and the WSXS motif. An example of an invariant amino acid residue is the W of the CXW (Trp$^\beta$) of the huEPOR motif in the class 1 receptors.

**FIG. 1.** Highly conserved amino acid residues of members of the cytokine receptor superfamily. A schematic representation of the extracellular domain of the huEPOR (where hu or h indicates human) is shown at the top of the figure. The extracellular domain of the huEPOR contains the cytokine receptor homology domain (CRH) which is predicted to fold into two motifs, designated the amino-terminal and carboxyl-terminal motifs (3). The alignment of cytokine receptor sequences indicates several conserved features across the receptor superfamily in both the class 1 and members of the class 2 receptors. Highly conserved amino acid residues are shown in bold; conserved motifs include the CXW, C$^\beta$-strand, boxed region of the F$^\beta$-strand, and the WSXS motif. An example of an invariant amino acid residue is the W of the CXW (Trp$^\beta$) of the huEPOR motif in the class 1 receptors.
highly conserved region in receptors of the cytokine receptor family (Fig. 1, Ref. 3). This amino acid conservation, along with the random mutagenesis results, led to the selection of this region for site-specific mutagenesis. Single alanine substitution mutants were made for all of the residues from Leu186 through Met200, located in the F' β-strand and in the loop between the E' and F' β-strands (see Fig. 2). The mutant EBP s were expressed in E. coli, and crude preparations were tested for EPO binding in the HP-SEC assay. In this assay, EBP bound to EPO is detected as a peak with a shorter retention time than that of EBP alone (Fig. 3; Ref. 16). All of the mutants spanning Leu186 through Met200 exhibited a specific EPO-EBP-bound peak in this assay, indicating that they were all capable of binding EPO (data not shown) and suggesting that none of the single alanine substitution mutants results in a 1,000-fold or greater reduction in EPO binding.

To assess the effects of mutations in this region, the EPO binding activity of selected mutants at both conserved and non-conserved residues of the F' β-strand was further characterized. The R189A-, R197A-, and M200A-EBP mutants were purified and assayed for EPO binding in the EBP-bead assay. The R189A and R197A mutations resulted in a 2–3-fold increase in the IC₅₀ value, whereas the M200A mutation had a greater effect on EPO binding, resulting in a 16-fold increase in the IC₅₀ value, whereas the M200A mutation had a greater effect on EPO binding, resulting in a 16-fold increase in the IC₅₀ value (Fig. 4B). The F55A-, Y57A-, and to some extent Y53A-EBP did not fold efficiently as judged by analytical HP-SEC experiments (Fig. 3). Although expression levels were normal, the F55A- and Y57A-EBP exhibited a relatively small peak at the retention time corresponding to that of the active wt EBP after 1 day of refolding. Protein in this peak did not accumulate with time (6 days of refolding), as was observed for the wt EBP and for EBP s with mutations in non-conserved residues in this region (Fig. 3). The small amount of F55A- and Y57A-EBP in this peak appeared to be active, as evidenced by the diminution of the peak upon the addition of excess EPO (Fig. 3); however, there was not enough protein present to purify and characterize further.

Despite reduced yields, sufficient amounts of the Y53A-EBP folded for purification and characterization of this mutant. The Y53A-EBP exhibited a 12-fold increase in IC₅₀ value relative to wt EBP in the EBP bead assay (Fig. 4B, Table I). The Q58A-EBP was purified in good yields and exhibited only a 4-fold increase in IC₅₀ value (Fig. 4B, Table I), even though this residue is immediately adjacent to the conserved Tyr²⁷, which when substituted with alanine had a dramatic effect on refolding and protein yield (Fig. 3). The C-D loop mutations in the non-conserved residues E60A and E62A resulted in mutant proteins that folded well (Fig. 3) and were purified in good yields. The E60A- and E62A-EBP increased IC₅₀ values in the EBP bead assay of 20- and 10-fold, respectively, suggesting that these residues may be involved in EPO binding (Fig. 4B, Table I). Comparable IC₅₀ values for the Q58A- and E60A-EBP were obtained in the inhibition of proliferation assay, confirming the effects of these mutations (Table I).

An examination of the cytokine receptor family alignment in Fig. 1 reveals that Trp⁴⁰ in the EPOR is analogous to an amino acid that is invariant in both the class 1 (CW) and class 2 (LXW) receptors, providing another conserved amino acid to
mutate. The W40A-EBP mutant did refold following expression in bacteria; however, the mutant protein did not appear to bind EPO in the HP-SEC assay or bound at a very low level (Fig. 5). The W40A-EBP was purified and subsequently tested in the EBP-bead and inhibition of proliferation assays. The IC50 value determined for the W40A-EBP was 0.8 μM for the EBP-bead assay (Fig. 4B, Table 1). An IC50 could not be determined for the W40A-EBP in the inhibition of proliferation assay, although the trend of the inhibition would suggest the IC50 to be greater than 1 μM (Table 1). These IC50 values are 130–160-fold higher than the wild-type EBP. The W40A mutation was then expressed as a full-length EPOR in COS cells and failed to bind EPO in the whole cell binding assay (Table I). Further investigation of this mutant full-length receptor utilizing a radiolabeled antibody cell surface detection method (15) revealed no detectable cell surface expression of the W40A full-length receptor (data not shown). These data suggest that W40A is a critical structural determinant of the EPOR.

Additional Trp residues that are conserved in these receptors are contained within the WSXWS motif, itself a conserved
feature of the cytokine receptors (Fig. 1). Previously reported data demonstrated that mutations to the WSXWS motif of the EPOR resulted in drastic effects on cell surface expression and ligand binding (22, 23). We chose to evaluate the effect of more conservative substitutions at the Trp residues of this motif. The W209Y and W212F mutants reported here retained an aromatic side chain moiety in these amino acid positions. These mutations had little effect on EPO binding, resulting in IC50 values increased by 8- and 3-fold for the W209Y and W212F mutants, respectively (Table I).

Several receptors in the cytokine receptor family have residues important for ligand binding located in the loop between the B and C β-strands (5, 12, 14). We have reported previously that Ser152 in the C β-strand of the EPOR may have a role in EPO binding, based on the 16-fold increase in IC50 value of the S152A-EPOR (15). Alanine substitution of Met150 and His153 in this loop resulted in slight increases in IC50 for EPO, indicating that these residues probably do not make a significant contribution to EPO binding. To expand our investigation of the function of residues in this region of the EPOR, we substituted alanine for the charged, aromatic, and polar residues (Arg155, Tyr156, Glu157, Asp159, and Ser161) in the predicted C β-strand (residues 154–161, see Fig. 2). These amino acids are not conserved in the cytokine receptor family, although an aromatic residue does appear to be maintained for many of the cytokine receptors at positions analogous to Tyr156. Crude preparations of these mutant EBPs bound EPO in the HP-SEC assay, indicating that none of these mutations resulted in a dramatic reduction (≥1,000-fold) in EPO binding (data not shown).

Chemical modification of the primary amines present in the EBP with NHS-biotin eliminates EPO binding (data not shown), suggesting that one or more lysine residues (Lys10, Lys14, Lys65) might contribute to ligand binding. Earlier work with truncated forms of the EBP showed that elimination of the first 10 amino acids of the EBP (including Lys10) had no gross effect on the ability of this mutant EBP to bind EPO. This mutant folds properly and is capable of binding EPO as analyzed by the HP-SEC assay (data not shown). A K14A full-length EPOR mutant expressed in COS cells demonstrated a Kd of 1.6 nM, which is a 3-fold increase relative to the wild-type EPOR (Table I). This relatively minor change in affinity suggested that if a lysine residue is involved with EPO binding it might be Lys65. The K65A-EBP had an IC50 value that was 20-fold higher than wild-type EBP, Fig. 4B, Table I. The non-conserved amino acids in the C-D loop together with Lys65 may represent a charge cluster essential for the binding of EPO to the EPOR.

**DISCUSSION**

In the present study, random mutagenesis was used to identify regions of the EBP that could be involved in EPO binding, based on non-biased placement of mutations within the protein. The original sequence alignment of the cytokine receptor

**TABLE I**

**EPO binding activity of mutations in the extracellular domain of the EPOR**

| Mutation | HP-SEC (+/-) | hEBP-bead | Inhib. prolif. | Receptor binding |
|----------|--------------|------------|----------------|-----------------|
| Wild-type| + 5          | 8          | 0.49           | 1               |
| K14A     | + 8          | 1.57       | NE†            | 3.2             |
| W40A     | – 800        | >1,000     |                | 160             |
| Y53A     | + 60         | –          |                | 12              |
| Q58A     | + 20         | 15         |                | 4               |
| E60A     | + 100        | 130        |                | 20              |
| E62A     | + 50         | –          |                | 10              |
| K65A     | + 100        | 110        |                | 20              |
| R189A    | + 15         | 18         | 0.45           | 3               |
| F194A    | + 12         | 0.33       | 1              |                 |
| V196A    | + 12         | 0.36       | 2.4            |                 |
| R197A    | + 12         | 0.32       | 1              |                 |
| M200A    | + 80         | –          | 16             |                 |
| W209Y    | + 40         | 180        |                | 8               |
| W212F    | + 15         | 30         |                | 3               |

a In cases where mutants were tested in more than one assay, the ratio reported is calculated from the EBP-bead assay data.

b — indicates mutant not tested in assay.

c Receptor was not expressed on the cell surface.
superfamily (3) identified conserved motifs and amino acid residues, and this alignment was used to select amino acids for site-directed mutagenesis (Fig. 1). Both approaches ultimately result in identifying amino acids that can be targeted by site-directed mutagenesis which can then help separate binding determinants from structural determinants. The random mutagenesis data implicated the F’ β-strand in ligand binding and led to an extensive analysis of this region of the EBP using site-directed mutagenesis. The HP-SEC assay was used to evaluate the binding characteristics of the F’ β-strand EBP mutants. Soluble M200A-EBP (lies at the beginning of the F’-G’ loop, Fig. 2) was tested in the EBP-bead assay and demonstrated a 16-fold increase in IC_{50} value (Table I). The recently solved crystal structure of the EBP with a peptide ligand (10) shows that Met^{200} is buried in the EBP but appears to be positioned below Phe^{205}, which is in proximity to the recently identified critical EPO binding determinant Phe^{205} (15, 24). The actual contribution of Met^{200} to ligand binding is most likely minimal; however, mutations of Met^{200} may exert some structural perturbations on the ligand binding face and alter the ability of this mutant EBP to bind EPO.

When selected F’ β-strand mutants were expressed as full-length EPOR constructs in COS cells, the binding affinities of these receptors were comparable with wild-type EPOR (Table I). However, receptor expression levels for both the R197A and R199A mutants as full-length EPOR were considerably lower than wild-type, suggesting that these mutations are having a structural effect on the receptor and are not true binding determinants for EPO. Amino acids that are highly conserved may be essential for the structural integrity of the protein, receptor folding, and trafficking to the cell surface, and therefore indirectly impact on ligand binding. The reduced receptor expression for Arg^{197} and Arg^{199} receptor mutants may be related to the interaction of these amino acids with the WSAWS motif, based upon the crystal structure of the EBP with a peptide ligand (10). The π-cation-π system found in the structure of the EBP relies on alternating stacking of two aromatic and two positively charged amino acid residues. This structure demonstrates the interactions of the Arg^{197}, Arg^{199}, and the WSAWS motif that are stabilized by main chain H bonds of Ser^{210} and Ser^{213} with the F’ β-strand. Further stabilization occurs through the formation of a salt bridge between Glu^{157} and Arg^{199} (10). Receptor cell surface expression for the full-length R189A, F194A, and V196A receptors was comparable with wild-type EPOR, most likely because these residues do not appear to be required for the structural stability of this region of the receptor. Therefore, mutations at selected amino acids of the F’ β-strand and WSAWS motifs of the EPOR may result in a destabilized receptor structure. The F’ β-strand, C’ β-strand, and the WSAWS motif are conserved sequences across the cytokine receptor superfamily (Fig. 1) (4). Mutations of the WSAWS box of the EPOR have been shown to have dramatic effects on cell surface expression due to retention of these receptors in the endoplasmic reticulum (23), although when one mutant receptor resulted in enhanced folding and surface expression (25). An extensive network of interactions exist in these areas of the EBP, and combined mutations may be necessary in order to destabilize this structure and observe a dramatic effect on binding.

The poor folding characteristics of mutants F55A-EBP and Y57A-EBP (Fig. 3) suggest that these conserved residues (Fig. 1) are important for structure, folding, and possibly protein processing. By day 6 very little refolded protein was observed, although the folded protein that was present was able to bind EPO in the HP-SEC assay, suggesting that these residues are involved in structural maintenance. Deletion of a short stretch of amino acids (QYFLY) in the C β-strand of the human GM-CSF receptor led to little surface expression with the majority of the mutant receptor accumulated intracellularly (26).

Analysis of the EBP crystal structure suggests that amino acid residues 60–63 (C-D loop, Fig. 1) may participate in the interaction with ligand (10), Glu^{50}, Glu^{62}, and Lys^{65} may represent a charge cluster that is necessary for interaction with EPO, and the substantial change in IC_{50} values for these mutants supports this proposal.

Single alanine substitutions in the C-D loop of the GHbp had only marginal effects on GH binding (14). However, alanine substitutions of residues within the C-D loop of the human IL-5Rα (D55, Y57) abolished IL-5 binding (27). The C-D loop of the EPOR may represent a hormone contact sequence conserved across the receptor superfamily. Alternatively, a structural motif may be present in the C-D loop that may also be a minor contact point with EPO.

The W40A-EBP did not demonstrate EPO binding in the HP-SEC assay (Fig. 5), but further characterization of W40A-EBP revealed an IC_{50} value of 800 pM in the EBP-bead assay. An IC_{50} could not be determined for the W40A-EBP in the inhibition of proliferation assay, although the trend of inhibition suggests a value of 1.0 μM or greater (data not shown). Trp^{40} may be a key structural determinant since it is invariant in the cytokine receptor superfamily (Fig. 1). Furthermore, the full-length W40A-EPOR did not demonstrate cell surface expression when assayed in COS cells. These data suggest that W40A is required for structural integrity and that the lack of cell surface expression indicates a perturbation of structure in this mutant protein. The W40A-EBP may not be stable over the course of the incubation time of the inhibition of proliferation assay. Moreover, the analogous residue in the growth hormone receptor, Trp^{50}, also showed a lack of cell surface expression (14) and, therefore, could not be characterized. The ability to produce a soluble W40A-EBP allows the characterization of a mutant receptor that would otherwise be unavailable for binding studies. Clearly, the bacterial expression, refolding, and purification of soluble EBP mutants have great utility in obtaining data from proteins that may be difficult to express.

The recently reported critical binding determinant of the EPO, Phe^{93}, exemplifies the impact that a single amino acid residue can have on ligand binding (15). The identification of
The except is Trp40 which had a 160-fold increase in IC50 points exist for the EPOR. Fine mapping of the EPO-EPOR evidenced by the disruption of protein folding and/or intracellular clearly involved in the structural integrity of the receptor, as the EPOR. Moreover, a number of amino acid positions are participate in more peripheral interactions between EPO and a moderate effect (10-fold or greater increase in IC50 value). Trp10 is located distal to the peptide binding face in the EBP crystal structure (10). The fact that a tryptophan is absolutely conserved at the analogous position in the receptor superfAMILY coupled with the lack of cell surface expression for receptors having mutations at this position suggests that this residue is essential for proper receptor structure. This conservation of amino acids also adds support to the prediction that all members of the cytokine receptor family have similar structures. The remainder of the EBP mutations showed little or no effect on EPO binding. The inability to find another single amino acid mutation that defines a critical binding determinant in this more extensive, albeit not exhaustive, search may not be surprising. Despite the fact that protein-protein interfaces involve extensive surface area interactions, it has been shown for the GH-GHbp complex that the vast majority of the individual intermolecular contact points make only minor contributions to the overall binding affinity. When the 33 GHbp residues shown to be involved in binding to site 1 of GH in the crystal structure of the GH-GHbp complex were examined by mutagenesis for individual contribution to the overall binding affinity, it was found that a subset of 11 contact residues formed the functional epitope (28). Among these 11 residues there were two for which mutation produced drastic reductions in binding affinity (14, 28).

The results of our mutational analysis of the EBP are consistent with the suggestion that a small number of residues comprise the critical binding determinants for such protein-protein interactions. We were unable to identify mutations either through random or site-directed mutagenesis that had as dramatic an effect on binding affinity as the previously identified Phe83 (15). Rather, we found that there were a number of residues with modest effects on binding that may in fact participate in more peripheral interactions between EPO and the EPOR. Moreover, a number of amino acid positions are clearly involved in the structural integrity of the receptor, as evidenced by the disruption of protein folding and/or intracellular trafficking. These data suggest that only a few key contact points exist for the EPOR. Fine mapping of the EPO-EPOR binding patch should permit attempts to identify a smaller functional epitope and aid in the rational design of small molecule ligands.

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