Identification of a Critical Ligand Binding Determinant of the Human Erythropoietin Receptor

EVIDENCE FOR COMMON LIGAND BINDING MOTIFS IN THE CYTOKINE RECEPTOR FAMILY*

(Received for publication, February 12, 1996, and in revised form, March 25, 1996)

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The erythropoietin receptor (EPOR) is a member of a family of cytokine and growth factor receptors that share conserved features in their extracellular and cytoplasmic domains. We have used site-specific mutagenesis within the extracellular domain of the EPOR to search for amino acid residues involved in erythropoietin (EPO) binding. Mutant proteins were expressed in bacteria as soluble EPO binding proteins (EBP) and characterized for EPO binding activity in a number of different assays. Substitution of phenylalanine at position 93 (Phe93) with alanine (F93A mutation) resulted in a drastic reduction in EPO binding in the EBP. More conservative tyrosine or tryptophan substitutions at Phe93 resulted in much less dramatic effects on EPO binding. Biophysical studies indicated that the F93A mutation does not result in gross structural alterations in the EBP. Furthermore, the F93A mutation in full-length EPOR expressed in COS cells abolished detectable EPO binding. This was not a result of processing or transport defects, since mutant receptor was present on the surface of the cells. Mutations in the region immediately around Phe93 and in residues homologous to other reported ligand binding determinants of the cytokine receptor family had small to moderate effects on EPO binding. These data indicate that Phe93 is a critical EPO binding determinant of the EPOR. Furthermore, since Phe93 aligns with critical ligand binding determinants in other receptors of the cytokine receptor family, these data suggest that receptors of this family may use common structural motifs to bind their cognate ligands.

Erythropoietin (EPO) is a glycoprotein hormone which functions as the primary regulator of erythropoiesis by inducing the survival and proliferation of erythroid progenitor cells and promoting their differentiation into mature red blood cells (1–3). EPO exerts these effects by binding a specific receptor (EPOR) on the surface of progenitor cells. The biology of EPO and its receptor have recently been reviewed (2, 4–6). The EPOR is found on erythroid progenitor cells and erythroid cell lines in relatively small numbers of between 300 and 1,000 per cell. In binding studies, EPOR has been detected on purified primary human erythroid progenitor cells with one affinity (Kd = 0.1 nM) or with two affinities (Kd = 0.1 and 0.57 nM) for 125I-EPO (7, 8). The biological significance of two affinities of EPO is unknown. Recent evidence suggests that there is an accessory component of the EPOR that increases EPO binding affinity (9–11), but this component(s) has yet to be identified. A single EPO binding affinity (Kd = 0.3–0.5 nM) has been reported for hematopoietic cell lines made EPO responsive by transfection with EPOR cDNA (12–14) and for transiently transfected COS cells (15).

Structurally, the human EPOR is a 484-amino acid glycoprotein having extracellular and cytoplasmic domains of about 225 amino acid residues each and a single membrane spanning domain (16). It is a member of an expanding family of cytokine and growth factor receptors that share homology in a region spanning approximately 200 amino acid residues of their extracellular domains designated the cytokine receptor homology (CRH) domain. Based on secondary structure predictions, Bazan (27) proposed that CRH domains contain two fibronectin type III-like motifs of seven $\beta$-strands each connected by a short "hinge" sequence. Thus, the tertiary fold of CRH domains was predicted to include two globular $\beta$-barrel domains of seven $\beta$-strands each with homology to fibronectin type III domains (17, 18). The crystal structures of the extracellular domains of ligand bound human growth hormone receptor (GHR), a member of the cytokine receptor family, and human tissue factor, a more distantly related family member, have confirmed these structural predictions (19, 20). Overall amino acid identity between CRH domains of members of the cytokine receptor family is generally less than 25%. However, alignment based on the predicted $\beta$-strands of the fibronectin type III-like motifs reveals several conserved features (17, 18, 21–23). These include two pairs of cysteines with conserved spacing in the amino-terminal motif, a WXSWS sequence near the carboxyl-terminal end of the membrane proximal motif, two highly conserved tryptophans, and several conserved tyrosines. Muta-
tional analysis of some or all of these conserved residues in several members of the cytokine receptor family, including the GM-CSFR (24, 25), IL-6R (26), IL-2R $\beta$ (27), GHR (28), prolactin receptor (29, 30), and EPOR (12, 13, 15, 31), suggests that these residues are primarily involved in stabilizing tertiary structure and/or in intracellular transport and protein process-
A Critical EPO Binding Determinant of the Human EPO Receptor

Amino acid residues important for ligand binding have been reported for a few members of the cytokine receptor family. For example, substitution of Trp104 of the GHR with alanine results in a >2,500-fold reduction in growth hormone (GH) binding (28). The importance of Trp104 was confirmed in the crystal structure of the GH bound GHR, in which Trp104 forms extensive van der Waals contacts with residues in GH (19). Based on crystallographic analyses, other important ligand binding contacts of the GHR are located in the loop between the B' and C' strands (B'-C' loop) in the membrane proximal motif of the CRH domain. Remarkably, the B'-C' loops of the human IL-2Rβ and the IL-3 specific second subunit of mouse IL-3R (AIC2A) also contain residues critical for binding their respective ligands (32, 33), and this same loop in the common β chain (βc) of the human GM-CSF, IL-3, and IL-5 receptors contains residues necessary for the formation of high affinity receptors (α chain-βc chain association) for GM-CSF and IL-5 (34). Recently, Arg188 of the human IL-5Rα, which is in a position homologous to Trp104 of the GHR, has been shown to be important for binding IL-5 (35). Thus, from these data, a pattern appears to be emerging in which receptors of the cytokine receptor family utilize different amino acid residues located in structurally homologous regions to bind their cognate ligands.

In this study, we have sought to identify EPO binding determinants on the EPOR by alanine replacement mutagenesis. Amino acid residues in the extracellular domain of the EPOR were chosen for mutagenesis based on their alignment with residues involved in ligand binding in other members of the cytokine receptor family. We have identified one residue, Phe93, that is a critical EPO binding determinant of the EPOR. Mutation of Phe93 to alanine results in an approximately 1,000-fold reduction in EPO binding in a competition binding assay and no detectable EPO binding in full-length mutant receptor expressed in COS cells. Substitution of Phe93 with leucine had a similar effect on EPO binding. More conservative substitutions with tyrosine or tryptophan had much less dramatic effects, suggesting that an aromatic residue is required at this position for high affinity EPO binding. Mutation of residues in the region immediately around Phe93 and residues homologous to other ligand binding determinants of the cytokine receptor family had small to moderate effects on EPO binding. Finally, Phe93 is homologous to Trp104 of the GHR and Arg188 of the IL-5Rα, both of which are involved in binding their cognate ligands (see above). Therefore, these results suggest that residues homologous to Phe93 in additional receptors of the cytokine receptor family are likely to be important for binding their respective ligands.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of the EBP—Mutagenesis was performed in a bacterial expression vector designed to produce a soluble EPO receptor (referred to here as an EBP binding protein or EBP) consisting of the 225 amino acid extracellular domain of the human EPOR truncated just prior to the beginning of the transmembrane domain. Construction of this vector and the expression, purification, and characterization of the EBP have been described previously (37). Briefly, the sequence encoding the EBP fused to the pb8 bacterial signal sequence was cloned into the T7 expression plasmid pET11a (Novagen, Inc., Madison, WI) and the completed construct was introduced into Escherichia coli strain BL21(DE3)pLyS5. EBP was produced in an insoluble form, solubilized, re-folded, and purified in yields of 0.6 mg per g of wet cell paste. NH2-terminal sequencing, peptide mapping, and mass spectrometry indicated that the purified EBP exhibited the expected amino terminus (signal sequence was properly cleaved off), disulfide bridging pattern of Cys59-Cys126 and Cys47-Cys103, and molecular mass. The E coli-expressed EBP exhibited a low nM EPO binding affinity (Kd = 5 nM), in the same range as the 1.5 nM Kd of a human EBP-glutathione S-transferase fusion protein expressed in bacteria (38) and the 1.1 nM Kd of a human EBP expressed in Chinese hamster ovary cells.

Mutations were created in the EBP expression vector described above using overlap-extension polymerase chain reaction (PCR) methods (41). Primers were designed to amplify a 515-base pair (bp) DNA fragment encoding a portion of the EBP from a unique XhoI site at residues 59–60 to a unique BamHI site just 3 bp downstream of the codon for residue 225. For each mutant construct, two primers, complementary to each other and encoding the desired mutation, were used in combination with the XhoI and BamHI primers to amplify two separate PCR fragments. These two fragments were combined in a second PCR reaction with the XhoI and BamHI primers to amplify the final 515-base pair PCR product containing the desired mutation. Mutant fragments were subsequently cloned into the unique XhoI and BamHI sites of the EBP expression vector and sequenced in entirety to confirm the presence of desired mutations and the absence of unwanted mutations.

Mutant EBPs were expressed in E. coli and purified as described for the wild-type EBP (37). Some mutant EBPs were purified by a modification of these methods in which the hydrophobic interaction chromatography (HI-SEC) step was replaced by a preparative high performance liquid chromatography (HPLC) step. The purity of mutant and wild-type EBP was estimated by SDS-polyacrylamide gel electrophoresis and analytical HP-SEC as described (37). The concentrations of purified mutant and wild-type EBP were estimated using the experimentally determined extinction coefficient for the wild-type EBP of 2.3 absorbance units per mg/ml at 280 nm (37).

Assay of the EPO Binding Activity of Wild-type and Mutant EBP—Assays in which mutant EBP was analyzed for the ability to compete for 125I-EPO binding with wild-type EBP immobilized on agarose beads (EBP bead assay) were performed as described (37). Analysis of EPO binding to wild-type and mutant EBP by HP-SEC was also performed as described (37). Inhibition of proliferation assessed using FDC-P1-tHER cells, a stably transfected cell line which proliferates in response to EPO. FDC-P1 cells (42), a hematopoietic IL-3 and GM-CSF responsive cell line, were made EPO responsive by transfection with a construct encoding a truncated human EPOR (tHER) lacking the carboxyl-terminal 40 amino acids of the cytoplasmic domain. This region is involved in down-regulation of EPOR signaling (43). The FDC-P1-tHER cell line was generated by methods described previously for FDC-P1 cells stably expressing mouse EPOR (14). The ability of mutant EBPs to inhibit the EPO-dependent proliferation of FDC-P1-tHER cells was assayed by standard methods, except that the assay was performed using a Biomek 1000 robotic workstation (Bedma Instruments, Inc., Fullerton, CA). Briefly, reservoirs were set up containing EBP alone and medium alone and used to prepare 96-well plates for the assay. Each well contained 40,000 cells, 0.25 ng/ml EPO, and a variable concentration of mutant EBP. Plates were incubated for 42 h at 37 °C, after which 1 μCi of [3H]thymidine (20 Ci/mmol, DuPont NEN, Boston, MA) was added per well, and incubation continued for an additional 6 h. Cells were collected onto Filtermat A glass fiber filters (Wallac, Inc., Gaithersburg, MD) using a Harvester 96 model Mach II cell harvester (TOMTEC, Inc., Orange, CT) and counts/min were determined using a Betaplate, model 1205 liquid scintillation counter (Wallac, Inc., Gaithersburg, MD). At the concentration of EPO used in the assay, the proliferative response of FDC-P1-tHER cells was just below maximal. For comparison with mutant EBPs, an inhibition of proliferation curve was generated with the wild-type EBP each time the assay was performed. In addition, a FDC-P1-tHER cell EPO response curve was generated each time the assay was run. All assays were performed in triplicate.

Biophysical Characterization of the Wild-type and F93A-EBP—The global structure of the wild-type and F93A-EBP was characterized using circular dichroism (CD) and fluorescence spectrometers. CD spectra were recorded using an Aviv 62DS spectrometer (Aviv Associates, Lakewood, NJ) equipped with a thermoelectrically controlled cell holder. The path length of the cuvette (Suprasil quartz cuvette, Helma Cells, Inc., Forest Hills, NY) was 1 mm. Each reported spectrum is baseline subtracted and an average of at least three scans. Fluorescence spectra were recorded employing an AVIV AT-105 Automated Titration/Difference Ratio Spectrofluorometer (Aviv Associates) equipped with a thermoelectrically controlled cell holder. The path length of the
A Critical EPO Binding Determinant of the Human EPO Receptor

FIG. 1. Alignment of residues of the human EPO R with residues implicated in ligand binding in other receptors of the cytokine receptor family. The upper part of the figure shows a representation of the extracellular region of the human EPO R (huEPR) which contains the cytokine receptor homology (CRH) domain. The CRH domain is predicted to fold into two motifs consisting of 7 \( \beta \)-strands each (17). The amino-terminal motif contains \( \beta \)-strands A through G and the carboxyl-terminal motif contains \( \beta \)-strands A' through G'. The CRH domain is further defined by four spatially conserved cysteines and a conserved WS motif. Residues of the CRH domain of the huEPR R are shown. TM designates the transmembrane domain. The lower part of the figure shows an alignment of residues in the E-F and G-C segments of the human (hu) and mouse (mo) EPR R with residues in the same \( \beta \)-strands of other receptors of the cytokine receptor family. The moEPR R differs from the huEPR R by only a few residues in the regions shown. The rat EPR R (Ref. 45, not shown) is identical to the moEPR R in these regions. Residue alignments are resolved by their \( \beta \)-strands as described by Bazan (17). The numbering of residues shown for the huEPR R begins with the first amino acid of the mature huEPR (16) or moEPR (46) and the numbering shown for the other receptors is taken from the references cited above.

cuvette (Suprasil quartz cuvette, Hellma Cells, Inc.) was 10 mm and emission and excitation slits were 5 nm.

Expression of Wild-type and F93A-EPO R in COS Cells—The DNA sequence encoding the human EPO R and the natural signal sequence (16) was subcloned into the mammalian expression vector pSG5 (Stratagene, La Jolla, CA). The F93A mutation created in the EBP expression vector (above) was subcloned into the pSG5-EPO R vector using EcoRI and BstXI restriction sites located within the sequence encoding the EBP and flanking Phe\(^{\text{B}}\). The presence of the F93A mutation in the pSG5-EPO R was confirmed by DNA sequencing.

COS-1 cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) plus 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) and 1% L-glutamine. All incubations were performed at 37\(^\circ\)C and 5% CO\(_2\). Cells were transfected by the DEAE-dextran/chloroquine diphosphate method. Prior to transfection, cells were detached from plates by a 5-min incubation with 0.05% trypsin in phosphate-buffered saline (PBS) and replated at the same density in DMEM containing 10% FBS. This step improved the yield of COS cells at harvest. Following an additional 24-h incubation at 37\(^\circ\)C, cells were washed with DMEM, overlaid with 10 ml of freshly prepared DNA-DEAE-dextran, and incubated overnight. Subsequently, cells were washed with DMEM, incubated 2.5 h with 10 ml of DMEM containing 10% FBS and 100 \( \mu \)M chloroquine diphosphate (Sigma), washed with DMEM again, and incubated 24 h with 10 ml of DMEM containing 10% FBS. Following this, cells were harvested for receptor binding assay and detection of receptor on the cell surface as described below.

Receptor Binding Assay and Detection of EPO R on the Surface of COS Cells—Equilibrium binding analyses were performed with \(^{125}\)I-EPO (100 \( \mu \)Ci/\( \mu \)l, DuPont NEN) essentially as described (44). Briefly, transfected COS cells were detached from plates by incubation at 37\(^\circ\)C for 20 to 60 min in 5 ml of PBS containing 0.5 mM EDTA and 0.02% sodium azide. Cells were washed with binding buffer (RPMI 1640, 25 mM HEPES pH 7.5, 5% bovine serum albumin, 0.02% sodium azide). Duplicate microcentrifuge tubes were set up with between 4 and 5 \( \times \) 10\(^{5} \) transfected cells. An appropriate concentration of \(^{125}\)I-EPO in 100 \( \mu \)l of binding buffer and a varied number of cells were incubated at 4\(^\circ\)C overnight. Cells were pelleted, resuspended in 100 \( \mu \)l of binding buffer, layered over 0.7 ml of calf serum, and pelleted again. The supernatant was removed by aspiration and the radioactivity in the pellet was determined by clipping the pellet into glass tubes and counting using a Micromedic \( \gamma \)-counter with an efficiency of 75% (ICN Micromedic, Huntsville, AL). Nonspecific binding was determined by the addition of 100-fold or greater excess unlabeled EPO. Specific binding was determined by subtracting counts/mi calculated from a linear fit of the nonspecific binding data from the total counts/min obtained. As a control, wild-type EPO R was assayed each time mutant EPO R was assayed.

Detection of EPO R on the surface of COS cells was performed using an anti-EPO monoclonal antibody (mAb) in a modification of the receptor binding assay described above. Duplicate microcentrifuge tubes were set up between 4 and 5 \( \times \) 10\(^{5} \) transfected cells in 100 \( \mu \)l of binding buffer to which 0, 0.1, or 1 \( \mu \)g of the immunoglobulin (Ig) fraction of anti-EPO mAb number 3 (Ref. 36, see below) in 100 \( \mu \)l of PBS. After overnight incubation at 4\(^\circ\)C, cells were pelleted and resuspended in 100 \( \mu \)l of binding buffer containing 0.1 \( \mu \)g (2 \( \mu \)Ci) of \(^{125}\)I-labeled sheep anti-mouse Ig (Amersham). Following a further 3-h incubation at 4\(^\circ\)C, cells were pelleted, resuspended in 100 \( \mu \)l of binding buffer, layered on 0.7 ml of calf serum, and pelleted again. The supernatant was removed and radioactivity in the pellet determined as in the receptor binding assay described above.

Production and Assay of Anti-EBP Monoclonal Antibodies—Mice were immunized with purified EBP (50 \( \mu \)g/mouse) emulsified with complete Freund’s adjuvant and boosted twice weekly with EBP (50 \( \mu \)g/mouse) emulsified with incomplete Freund’s adjuvant. Antibody reactivity to EBP was monitored by enzyme-linked immunosorbent assay (see below). Hybridomas were prepared by Maine Biotechnology Services, Inc. (Portland, ME) using standard methods.

The binding of mAbs to EBP was measured in an enzyme-linked immunosorbent assay format. The Ig fraction of a rabbit anti-EBP polyclonal antisera (0.1 \( \mu \)g in PBS) raised against purified EBP was adsorbed to microtiter plates and wells were blocked with 1% bovine serum albumin in PBS. EBP (0.01 \( \mu \)g in PBS) was added and the plates were incubated overnight at 4\(^\circ\)C. Following a wash with PBS, 0.05% Tween 20, mAb was added, and plates were incubated at room temperature for 2 h. Binding of mAb to the EBP was detected by the addition of an anti-mouse peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and measuring the absorbance at 450 nm.

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RESULTS

Mutagenesis Strategy—We have previously described a system for the overexpression of the extracellular domain of the human EPOR (termed EPO binding protein or EBP) in E. coli (Ref. 37, see "Experimental Procedures"). The E. coli expressed EBP can be purified in milligram quantities and binds EPO with a 5 nM affinity, making it suitable for mutagenesis studies. Amino acid residues in the EBP were selected for substitution with alanine based on their alignment with residues known to be involved in ligand binding in related receptors of the cytokine receptor family. In published mutagenesis studies at the time this work was initiated, residues in the E-F loop of the human GHR and in the B\(^9\)-C\(^9\) loops of the human IL-2R\(_b\) and mouse IL-3R (AIC2A) were reported to be involved in binding their respective ligands (see Fig. 1). Therefore, residues in homologous positions in the EBP were targeted for mutagenesis. Our strategy was to mutate these selected residues to alanine, express the mutant EBPs in E. coli, and test crude preparations of mutant EBP for EPO binding in an HP-SEC assay (see below). Using this assay we could quickly and easily determine whether or not the mutant EBPs still bound EPO with reasonable affinity. Interesting mutant EBPs identified by these methods could then be purified and their EPO binding activity further characterized utilizing several different assays.

The F93A Mutation Results in a Dramatic Reduction in EPO Binding in the EBP—The loop between the E and F \(\beta\)-strands (E-F loop) of the GHR contains a tryptophan (Trp\(^{104}\)) which has been shown to be crucial for ligand binding (19, 28). The homologous position in the predicted E-F loop of the EPOR is occupied by a phenylalanine, Phe\(^{93}\) (see Fig. 1). To investigate the possibility that this residue is involved in binding EPO, Phe\(^{93}\) in the EBP was replaced with alanine (F93A mutation). The F93A-EBP was initially checked for EPO binding activity utilizing HP-SEC. In this assay, EBP bound to EPO is detected as a peak with a shorter retention time than that of EBP alone (Fig. 2A). As shown in Fig. 2B, purified F93A-EBP appears as a peak with a similar retention time to that of wild-type EBP but, unlike wild-type EBP, does not form a detectable EPO-bound peak in the presence of excess EPO. Since the HP-SEC analysis suggested that the F93A-EBP does not bind EPO, we further characterized the effect of this mutation on EPO binding in the EBP bead and inhibition of proliferation assays. In the EBP bead assay, EBP (wild-type or mutant) competes for \(^{125}\)I-EPO binding with EBP immobilized on agarose beads (37) while, in the inhibition of proliferation assay, EBP competes for EPO binding with EPOR on the surface of a stably transfected, EPO responsive cell line (see "Experimental Procedures"). The F93A-EBP bound EPO in these assays, but binding was detectable only at micromolar concentrations of added F93A-EBP (Fig. 3). The F93A-EBP exhibited an IC\(_{50}\) of \(\sim 5,000\) nM in the EBP bead assay, a 1,000-fold increase over the wild-type EBP (see Table I). An IC\(_{50}\) value could not be calculated from the inhibition of proliferation data, however, because the F93A-EBP could not be added to the assay at the concentrations needed to completely inhibit proliferation (Fig. 3B).

To more thoroughly investigate the function of Phe\(^{93}\) in EPO binding, additional amino acid substitutions were made at this position in the EBP. Phe\(^{93}\) was replaced with tyrosine (F93Y) and tryptophan (F93W) which are more conservative substitutions for phenylalanine than the alanine (F93A) substitution. In addition, Phe\(^{93}\) was replaced with leucine (F93L) which has a larger hydrophobic side chain than alanine. The F93Y and F93W mutations had relatively small effects on the EPO binding activity of the EBP compared to the F93A mutation (Fig. 3), resulting in increased IC\(_{50}\) values of 8- and 60-fold, respectively, relative to wild-type in the EBP bead assay (see Table I). The F93L-EBP, however, exhibited greatly reduced EPO binding activity similar to the F93A-EBP (Fig. 3). These data sug-

Fig. 2. Assay of the EPO binding activity of the wild-type and F93A-EBP by HP-SEC. The HP-SEC analyses were performed as described previously (37). Purified wild-type EBP appears as a single peak with a retention time of about 9.5 min (A, solid trace). In the presence of excess EPO, the EBP peak is completely converted to an EPO bound peak with a retention time of about 8.3 min (A, dashed trace). The purified F93A-EBP also appears as a single peak with a retention time of about 9.5 min (B, solid trace) but is not converted to an EPO bound complex in the presence of excess EPO (B, dashed trace). Slightly more F93A-EBP was added in the plus EPO run (B, dashed trace) so that the peak at 9.5 min would be visible.
suggest that the aromatic functionality of Phe93 may be important for EPO binding and that Phe93 is a critical ligand binding determinant of the EPOR. In addition, a double mutant was created in which Phe93 was replaced with alanine and the serine adjacent to Phe93 (Ser92) was replaced with phenylalanine (S92F-F93A EBP). The S92A mutation alone resulted in a relatively modest effect on EPO binding (see below), while, even at concentrations of 5 μM, the double mutant did not exhibit enough EPO binding activity in the EBP bead assay to permit calculation of an IC50 (data not shown). This suggests that the precise location of an aromatic residue at position 93 is important for EPO binding.

The F93A Mutation Does Not Result in Gross Structural Changes in the EBP—To explore the possibility that the reduced EPO binding of the F93A-EBP is a result of structural changes caused by the mutation and not the absence of a critical EPO binding determinant, we probed the structure of the F93A-EBP using several different methods. Initial evidence that the F93A mutation does not perturb the overall structure of the EBP was provided by the HP-SEC analysis. As can be seen in Fig. 2, the purified F93A-EBP appears as a sharp peak with a retention time identical to that of the wild-type EBP, suggesting that the mutant EBP has a hydrodynamic volume similar to that of wild-type. The global structure of the F93A-EBP was further evaluated using a panel of anti-EBP mAbs. Anti-EBP mAbs were generated as described under "Experimental Procedures" and characterized for their ability to bind EBP. Four mAbs were selected which bind non-overlapping epitopes on the EBP with low nanomolar affinity (data not shown). Three of the mAbs (mAb numbers 3, 4, and 7) bound to the F93A-EBP and wild-type EBP equally well (Fig. 4), suggesting that the F93A mutation does not cause gross structural changes in the EBP. One of the anti-EBP mAbs (mAb number 9) bound the F93A-EBP only half as well as wild-type EBP (Fig. 4). Importantly, mAb number 9 was the only mAb isolated that was capable of completely inhibiting (neutralizing) the binding of EPO to cell surface EPOR in a proliferation assay (data not shown), suggesting that this mAb may bind an epitope(s) on the EBP involved in EPO binding.

As an additional measure of the possible structural effects of the F93A mutation, we evaluated the overall secondary structure of the F93A-EBP relative to wild-type using circular dichroism (CD). The F93A mutation resulted in virtually no changes in the CD spectrum of the EBP (Fig. 5), suggesting that the F93A mutation does not alter the global secondary structure of the EBP. This conclusion is further supported by a comparison of the thermal stabilities of the F93A-EBP and wild-type EBP as measured by CD and tryptophan fluorescence. The mutant and wild-type EBP exhibit nearly identical Tm values in thermal transitions measured by CD (Fig. 6A) and tryptophan fluorescence (Fig. 6B). Thus, the F93A mutation does not ap-

![Fig. 3. Effects on EPO binding of a series of mutations at Phe93 of the EBP.](http://www.jbc.org/)

![Fig. 4. Assessment of the overall structure of the F93A-EBP relative to wild-type using a series of anti-EBP monoclonal antibodies.](http://www.jbc.org/)
pear to alter the thermal stability of the secondary structures or the local environments of the 6 tryptophans of the EBP. Taken together, the data presented above indicate that the F93A mutation does not result in large structural alterations in the EBP.

The F93A Mutation Results in a Dramatic Reduction in EPO Binding in Full-length EPOR Expressed in COS Cells—The data presented above indicate that the F93A mutation causes a dramatic reduction in EPO binding in the bacterially-expressed soluble EBP. To determine if the F93A mutation has a similar effect on full-length cell surface EPOR, it was subcloned into a mammalian EPOR expression vector and expressed in COS cells (see "Experimental Procedures"). The wild-type EPOR expressed in COS cells showed binding that was saturable at about 20 nM $^{125}$I-EPO (Fig. 7). The calculated $K_d$ of the wild-type was 0.37 nM, in good agreement with previously reported values of between 0.3 and 0.5 nM for the EPOR expressed in COS cells (15). The F93A-EPOR, however, did not exhibit detectable binding, even at $^{125}$I-EPO concentrations as high as 90 nM (Fig. 7). Attempts to detect binding activity for the F93A-EPOR using more concentrated $^{125}$I-EPO, permitting the addition of up to 90 $\mu$M $^{125}$I-EPO to the assay, were unsuccessful due to high background and reduced signal (data not shown). The lack of detectable EPO binding observed for the F93A-EPOR could be a result of the mutant receptor not being present on the surface of the transfected COS cells. To investigate this possibility, we used an assay in which mouse anti-EBP mAb number 3 was bound to cell surface EPOR and detected with $^{125}$I-labeled sheep anti-mouse Ig (see "Experimental Procedures"). As can be seen in Fig. 8, COS cells transfected with the wild-type or F93A-EPOR exhibited an mAb number 3-dependent signal over cells with no DNA transfected. Moreover, this signal was reduced to background in the presence of excess competing EBP, demonstrating that specific counts/min in this assay are dependent on mAb number 3 binding to the COS cells. These results indicate that the apparent loss of EPO binding activity in the F93A-EPOR does not result from cell transport or processing defects caused by the mutation, since the mutant EPOR was present on the surface of cells in numbers similar to wild-type (Fig. 8).

Mutations in the Region Adjacent to Phe$^{93}$ and in the B'C' Loop Region Result in Relatively Small Effects on EPO Binding—To determine if residues adjacent to Phe$^{93}$ in the pre-

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**Fig. 5.** CD spectra of the wild-type and F93A-EBP. CD spectra were generated at 25°C and 0.365 mg/ml protein in PBS buffer as detailed under "Experimental Procedures." The F93A mutation results in essentially no change in the CD spectrum of the EBP.

**Fig. 6.** Thermal stability of the F93A-EBP compared to wild-type. Spectra for the CD melting curves were recorded at various temperatures using 0.365 mg/ml protein in PBS. CD melting curves were generated by plotting the ellipticity obtained at 228 nm, the wavelength which provided the widest differences in ellipticity over the temperature range. Tryptophan fluorescence was measured at an excitation wavelength of 220 nm and an emission wavelength of 350 nm using 3.39 $\mu$g/ml protein in PBS. The thermal melting temperature ($T_m$) measured by circular dichroism (A) and tryptophan fluorescence (B) of the F93A-EBP is nearly identical to that of wild-type. The key for the symbols used in both A and B is given in A.

**Fig. 7.** $^{125}$I-EPO equilibrium binding analyses of COS cell-expressed wild-type and F93A-EPOR. Receptor binding assays were performed as described under "Experimental Procedures." Total counts/min (●), specific counts/min (○), and nonspecific counts/min (×) are shown for the wild-type EPOR and specific counts/min (□) are shown for the F93A-EPOR. Binding to the wild-type EPOR was saturated at about 20 nM $^{125}$I-EPO while no binding was apparent for the F93A-EPOR out to 90 nM $^{125}$I-EPO. The inset shows a Scatchard plot of the data for the wild-type EPOR. The $K_d$ for $^{125}$I-EPO derived from the wild-type binding data was 0.37 nM.
Thr90, Ser91, Ser92, and Val94 in this loop were replaced with 
directed E-F loop of the EBP are also involved in EPO binding, 
Thesis and Ser92, and Val94 in this loop were replaced with alanine (see Fig. 1). The mutant proteins were expressed in E. coli, purified, and tested for EPO binding in the EBP bead assay and inhibition of proliferation assays. The data shown in Fig. 9 and summarized in Table II indicate that these mutations do not result in large reductions in EPO binding in the EBP. Mutation of the residues on either side of Phe93 (Ser92 and Val94) to alanine resulted in 10- and 16-fold increases in IC_{50} respectively, suggesting that these residues may be involved in EPO binding. However, these increases are small in comparison to the 1,000-fold increase in IC_{50} resulting from the substitution of Phe93 with alanine.

Two additional residues, Leu96 and Glu97, in the predicted F 
(b-strand of the EBP (Fig. 1) were also replaced with alanine. 
While the E97A-EBP possessed nearly wild-type EPO binding 
activity, the L96A-EBP showed a considerable reduction in 
EPO binding in the EBP bead assay (data not shown), exhibiting a 200-fold increase in IC_{50} relative to wild-type (Table II). 
Thus, Leu96 may be an important ligand binding determinant 
of the EPOR. However, despite similar expression levels, the 
L96A-EBP was purified in low yields after the re-folding step 
compared to the wild-type and other mutant EBPs, suggesting 
that the L96A-EBP does not fold efficiently (data not shown). 
We do not know what effect this mutation might have on the 
structure of the EBP, since the CD spectra, antibody binding 
profile, and thermal denaturation properties of this mutant 
were not determined in this study. 

Several receptors in the cytokine receptor family have resi 

dues important for ligand binding located in the loop between 
their B- and C-strands (B-C loop, see Fig. 1). The amino 
acid residues predicted to be in the B-C loop of the human 
EPOR are LPPPETPTMST (Fig. 1). The function that the 4 
prolines of this loop may have in EPO binding was not inves 
tigated because interpretation of the results would have been 
complicated by the structural contributions of the proline resi 
dues. The functions of the glutamate and first threonine in this 
loop were also not investigated because the homologous resi 
dues in the mouse EPOR are glycine and alanine, respectively 
(Ref. 46 and Fig. 1), and it is likely that residues providing a 
significant contribution to EPO binding would be conserved 
across species. This leaves the last four residues of the B'-C' 
loop (Met150-His153) as the most likely candidates to function in 
EPO binding. Of these residues, we replaced Met150, Ser152, 
and His153 with alanine (Fig. 1). Initial attempts to replace 
Thr151 with alanine failed and mutagenesis of this residue was 
not pursued further. To examine additional residues in the 
region adjacent to the B'-C' loop, Arg151 in the predicted B' 
(b-strand and Arg155 in the predicted C' b-strand of the 
EBP were replaced with alanine (see Fig. 1). The mutant proteins 
were expressed in E. coli, purified, and tested for EPO binding 
in the EBP bead and inhibition of proliferation assays (data not 
shown). The IC_{50} values derived from these data indicate that 
these mutations do not have a substantial effect on EPO binding 
(Table II). One mutation, S152A, results in a 220-fold increase in 
IC_{50}, suggesting that Ser152 may be involved in EPO binding. 
However, if Ser152 is involved in binding EPO, its 
contribution is modest compared to Phe93.

**DISCUSSION**

In this study, we have determined that Phe93 in the loop 
between the predicted E and F b-strands (E-F loop) of the 
EPOR is crucial for EPO binding. The F93A mutation results in 
a dramatic reduction in EPO binding in a bacterially-expressed 
soluble EBP. Moreover, the F93A mutation causes a loss 
of detectable binding in full-length EPOR transiently expressed 
in COS cells. This mutation apparently does not affect trans 
port or protein processing of the EPOR in COS cells, since
A Critical EPO Binding Determinant of the Human EPO Receptor

TABLE I

IC_{50} data for EBP mutations in the regions of the E-F and B'-C' loops

| Mutation | IC_{50}^{mut} | IC_{50}^{wt} | IC_{50}^{mut}/IC_{50}^{wt} |
|----------|---------------|--------------|--------------------------|
| E - F loop region mutants |
| Wild-type | 5 | 8 | 1 |
| T90A | 30 | 17 | 2 |
| S91A | 25 | 22 | 1 |
| S92A | 50 | 100 | 0.5 |
| F93A | 5,000 | — | 1,000 |
| V94A | 80 | — | 16 |
| L96A | 1,000 | — | 200 |
| E97A | 40 | 30 | 8 |
| B - C' loop region mutants |
| Wild-type | 5 | 8 | 1 |
| R141A | 20 | 20 | 1 |
| M150A | 20 | 15 | 1.3 |
| S152A | 50 | 80 | 0.62 |
| H153A | 15 | ND | 3 |
| R155A | 40 | 50 | 8 |

a Derived from EBP bead competition binding data.
b Derived from inhibition of proliferation data.
c Not done.
d Mutant did not exhibit enough activity in this assay for calculation of an IC_{50} value.

The reduced EPO binding activity of the F93A-EBP is not a result of gross conformational changes based on the following observations. First, the purified F93A-EBP formed a sharp peak and exhibited a retention time identical to that of wild-type in HP-SEC experiments. This suggests that the F93A-EBP exists as a single species, rather than a collection of folding intermediates, and possesses a hydrodynamic volume similar to that of wild-type. Second, the binding of a series of anti-EBP mAbs to the EBP was unaffected by the presence of the F93A mutation, suggesting that the global conformation of the F93A-EBP is unchanged relative to wild-type. However, one mAb (mAb number 9) exhibited reduced binding to the F93A-EBP relative to wild-type, indicating that part of its binding epitope on the EBP is disrupted by the F93A mutation. Results from inhibition of proliferation experiments indicated that mAb number 9 is the only mAb of the series capable of neutralizing the binding of EPO to cell surface EPOR. This suggests that mAb number 9 may interact with the EPO binding site of the EBP, including, perhaps, Phe93. Thus, it is possible that the reduced binding of mAb number 9 to the F93A-EBP is due to the absence of Phe93 itself, rather than structural perturbations caused by the mutation. Taking into account all of the data presented here, this seems to be the most plausible explanation of the results for mAb number 9. Third, the CD spectra of the wild-type and F93A-EBP are almost identical, suggesting that the F93A mutation does not cause gross changes to the secondary structure of the EBP. Finally, the thermal denaturation temperature (T_m) of the F93A-EBP is identical to that of wild-type, as measured by CD and tryptophan fluorescence. These results indicate that the F93A mutation does not alter the relative stabilities to heat denaturation of either the secondary structural elements or the environments around the tryptophans of the EBP. In addition, the fact that the T_m values in these experiments are the same regardless of whether Phe93 is present or substituted with alanine suggests that this residue is not involved in stabilizing the global structure of the EBP. Taken together, the results presented above provide strong evidence that gross structural changes are not responsible for the dramatic reduction in EPO binding observed for the F93A-EBP.

Compared to the mutation of other residues in the E-F loop region of the EBP, the F93A mutation results in a major reduction in EPO binding. This suggests that Phe93 acts as a discrete contact point for interaction with EPO rather than as part of a binding surface in which several residues contribute to ligand binding. Mutation of Ser92 and Val93 to alanine results in 10- and 16-fold increases in IC_{50} values, respectively, indicating that these residues may have a role in EPO binding. Alternatively, the increased IC_{50} values resulting from these mutations may be due to indirect effects on the position or orientation of Phe93 rather than the loss of the side chains themselves. Whichever is the case, the effects of mutations in the other residues of the E-F loop are small compared to that of the F93A mutation. Thus, Phe93 appears to be the only residue in the predicted E-F loop of the EPOR that makes a significant contribution to EPO binding. Alanine substitution of Leu96 in the F beta-strand resulted in a 200-fold increase in IC_{50} relative to wild-type EBP, suggesting that Leu96 is involved in EPO binding. However, alignment of receptors in the cytokine receptor family indicates that Leu96 is in a position homologous to a highly conserved tyrosine (17, Fig. 1), suggesting that this residue is important for folding or structure in the EPOR. Mutation of highly conserved residues in several different receptors of the cytokine receptor family has shown that these residues are important for folding, structure, and/or cellular processing (24-31).

Alignment of the predicted beta-strands of the GHR and EPOR indicates that Trp104 in the E-F loop of the GHR is homologous to Phe93 of the EPOR (Ref. 17 and see Fig. 1). Mutagenesis results reported for Trp104 are strikingly similar to those reported here for Phe93. Substitution of Trp104 with alanine resulted in a greater than 2,500-fold reduction in GH binding, based on the increased K_d of the mutant receptor (28). Mutation of the other residues in the E-F loop of the GHR had minimal effects on GH binding, resulting in no more that a 2-fold increase in K_d. None of the many other residues of the GHR that were examined exhibited anywhere near as great a contribution to GH binding as did Trp104, with the next largest effect being an 85-fold increase in K_d for substitution of Pro106 with alanine (28). Thus, like Phe93 of the EPOR, Trp104 of the GHR is located in the E-F loop, its substitution with alanine results in a dramatic decrease in ligand binding, and surrounding residues do not make a significant contribution to ligand binding.

Based on the location of ligand binding determinants in other receptors of the cytokine receptor family, we investigated the possibility that the B'-C' loop region of the EPOR contained residues involved in EPO binding (see Fig. 1). Of the mutations made in this region, S152A had the largest effect, resulting in a 16-fold decrease in EPO binding based on the IC_{50} of the mutant protein in the EBP bead assay. Notably, the residue...
homologous to Ser^{152} in the mouse EPOR is a threonine (46, Fig. 1), suggesting that the hydroxyl functionality at this position is conserved. Together with the decrease in EPO binding observed in the S152A-EBP, this suggests that Ser^{152} is an EPO binding determinant of the EPOR. However, the contribution of Ser^{152} to EPO binding is minor relative to that of Phe^{93} in the E-F loop. Based on these results, the B'-C' loop of the EPOR does not appear to contain residues having a significant role in EPO binding. It is possible, however, that Thr^{151} of this loop is involved in EPO binding, since we did not investigate the role of this residue.

In contrast to the results reported here for the EPOR, the B'-C' loops of several receptors of the cytokine receptor family have been reported to contain residues crucial for ligand binding. In particular, residues in this loop in the human IL-2Rβ and the IL-3 specific mouse IL-3R (AIC2A) have been shown to be critical for ligand binding (Refs. 32 and 33, see Fig. 1). Mutations of multiple residues within these sequences abolished ligand binding, however, mutant receptors with single alanine substitutions within these sequences bound ligand with relatively modest reductions in affinity of 4–17-fold. This suggests that these residues act in concert to form a binding surface for contact with ligand or a conformationally unique structure in which the individual residues are not as important as all of the residues together. We do not know if this is also the case for residues in the B'-C' loop of the EPOR, since we only made single alanine substitution mutants in this study. Recently, three residues in the B'-C' loop of the common β chain (βc) of the human GM-CSF, IL-3, and IL-5 receptors have been shown to be critical for the formation of the high affinity receptors for GM-CSF and IL-5 (Ref. 34 and see Fig. 1). High affinity binding was abolished whether these residues were replaced with alanine individually or as a group. Thus, as was the case for the human IL-2Rβ and the mouse IL-3R, several residues in the B'-C' loop of the human βc form a binding surface for contact with ligand. In this case, however, each of the individual residues of the binding surface appear to be critical for binding.

The presence of important ligand binding determinants in the E-F and/or B'-C' loops of several different cytokine receptors reveals a pattern in which these receptors utilize residues in structurally homologous locations to bind their cognate ligands. For the EPOR and GHR, a residue in the E-F loop appears to mark the most significant contacts with ligand, although a role for residues in the B'-C' loop cannot be ruled out. Crystallographic results indicate that, in addition to the contacts with Trp^{104} in the E-F loop, GH forms extensive interactions with several residues (especially Trp^{169}) in the B'-C' loop of the GHR (19). Although these residues have not been examined by site-specific mutagenesis, the structural data provide compelling evidence that both the E-F and B'-C' loops of the GHR are involved in GH binding. Recently, a residue in the E-F loop of the human IL-5Rα has been shown to contribute to the interaction with IL-5 (Ref. 35 and Fig. 1). Substitution of this residue with alanine resulted in a greater than 100-fold reduction in IL-5 binding, based on the Kd of the mutant IL-5Rα. Thus, the high affinity receptor complex composed of the IL-5Rα and βc subunits also appears to utilize residues in the E-F and B'-C' loops for ligand binding, since IL-5 interacts the E-F loop of the IL-5Rα and the B'-C' loop of the βc (Refs. 34 and 35, see above). For both the human IL-2Rβ and mouse IL-3R (AIC2A) residues in the B'-C' loop have been shown to interact with ligand (Refs. 32 and 33, see above). It is not known if residues in the E-F loops of these receptors are involved in ligand binding, since mutations in the E-F loops of these receptors have not been reported. The high affinity receptors for these cytokines might be similar to the high affinity IL-5 receptor, having residues in the E-F loops of their respective α-chains that contact ligand.

Our results, identifying a critical ligand binding determinant located in the E-F loop of the EPOR, provide additional evidence for the ligand binding paradigm described above for receptors of the cytokine receptor family. This is not to say that ligand contacts occur only in the E-F and B'-C' loops in receptors of this family. We have investigated the function of over 40 residues outside of these loops in the EBP, some of which show as much as 20-fold increased IC_{50} values for EPO and may be involved in EPO binding. In addition to residues within the E-F and B'-C' loops, residues involved in ligand binding outside of the E-F loops have been identified for the mouse IL-3R (AIC2A, Ref. 33) and human IL-5Rα (35). Residues located outside of the E-F and B'-C' loops have been shown to be important for ligand binding in the rat prolactin receptor (29), however, residues within these loops were not investigated. Data from structural and mutagenesis studies of additional receptors of the cytokine receptor family are needed before we can say for certain that the presence of ligand binding determinants in the E-F and B'-C' loops of these receptors is a general phenomenon.

The results presented here provide functional information on the interaction of EPO with its receptor, however, a high resolution structure of the EPO-EPOR complex is necessary to confirm the molecular details of this interaction. Livnah et al. have generated a high-resolution crystal structure of the EBP bound to an EPO mimic peptide. The EPO mimetic peptide forms major contacts with both the E-F and B'-C' loops of the EBP, providing structural evidence supporting the functional data presented here for EPO binding to the EBP. In conclusion, our mutational analysis of the EPOR, combined with high resolution structural data, should provide information useful in the design of small molecule mimics of EPO.

Acknowledgments—We are grateful to Greg Price and Mark Koehler for high-density fermentation of several of the mutant EBPs and Wise Lumax for purification of the S152A-EBP. We are also grateful to Adriane Schilling for DNA sequencing, and to Woan-Hwa Lee for monoclonal antibody work.

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A Critical EPO Binding Determinant of the Human EPO Receptor

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