We have shown previously that Phe93 in the extracellular domain of the erythropoietin (EPO) receptor (EPOR) is crucial for binding EPO. Substitution of Phe93 with alanine resulted in a dramatic decrease in EPO binding to the Escherichia coli-expressed extracellular domain of the EPOR (EPO-binding protein or EBP) and no detectable binding to full-length mutant receptor expressed in COS cells. Remarkably, Phe93 forms extensive contacts with a peptide ligand in the crystal structure of the EBP bound to an EPO-mimetic peptide (EMP1), suggesting that Phe93 is also important for EMP1 binding. We used alanine substitution of EBP residues that contact EMP1 in the crystal structure to investigate the function of these residues in both EPOR and EMP1 binding. The three largest hydrophobic contacts at Phe93, Met150, and Phe205 and a hydrogen bonding interaction at Thr151 were examined. Our results indicate that Phe93 and Phe205 are important for both EPO and EMP1 binding. Met150 is not important for EPO binding but is critical for EMP1 binding, and Thr151 is not important for binding either ligand. Thus, Phe93 and Phe205 are important binding determinants for both EPO and EMP1, even though these ligands share no sequence or structural homology, suggesting that these residues may represent a minimum epitope on the EPOR for productive ligand binding.

Erythropoietin (EPO),1 the primary cytokine involved in the regulation of red blood cell production, functions by binding to a cell surface receptor (EPOR) on red blood cell precursors (1–5). Signaling through the EPOR includes the activation of a receptor-associated tyrosine kinase, JAK2 (3), which leads to the subsequent phosphorylation and activation of STAT5 (4, 5), a member of the signal transducer and activator of transcription (STAT) family of transcriptional activators (reviewed in Ref. 6). Activated STAT5 moves to the nucleus where it promotes transcription of target genes by binding to specific STAT5 response sequences. In binding studies, the 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 nM and 0.57 nM) for 125I-EPO (7, 8). The significance of two affinities of the EPOR for EPO is not clear. 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. Alternatively, two affinities of the EPOR for EPO may arise from two nonequivalent receptor binding sites on EPO, one of high affinity (–1 nM) and one of low affinity (–1 μM), that have been reported for the extracellular domain of the EPOR in solution (12). Structurally, the human EPOR is a 484-amino acid glycoprotein with a single transmembrane segment located between extracellular and intracellular domains each of nearly equal size (13). EPOR is a member of a large family of cytokine and growth factor receptors whose ligand binding domains contain homologous sequences and are predicted to be structurally related (14). These ligand binding domains consist of approximately 200–250 amino acid residues and contain two subdomains, each predicted to consist of seven β-strands and to be structurally related to fibronectin type III (FNIII) domains (14, 15). The amino-terminal FNIII-like domain contains a pair of spatially conserved cysteine bridges, while the carboxyl-terminal FNIII-like domain contains a conserved β-strand F and a highly conserved WSXWS motif that are hallmarks of receptors of the cytokine receptor family (14).

Previously, we reported the characterization of an EPO-binding protein (EBP), consisting of amino acids 1–225 of the extracellular domain of the mature human EPOR, that was expressed in E. coli in quantity and purity sufficient for site-specific mutagenesis and crystallographic studies (16). Recently, the crystal structures of the EBP bound to agonist peptide (EPO-mimetic peptide, EMP1) and an antagonist peptide (EMP33) have been determined (17, 18). EMP1 is a 20-amino acid peptide that is one of a series of related peptides discovered by phage display methodology (19). EMP1 exhibits no homology to EPO, yet binds specifically to the EPOR and mimics the biological effects of EPO both in vitro and in vivo (19). The structure of the EBP in the EMP1-EBP complex...
consists of two domains, each containing seven β-strands arranged in a FNIII-like topology (17). This topology for the EPOR was also observed in the recently described crystal structure of EPO and EMP1 binding to EPO (20). The structure of the EPOR shows close homology with the structures of ligand binding domains of GHR, prolactin receptor, granulocyte colony-stimulating factor receptor, and GP130 (21–24), all members of the cytokine receptor family. The EPOR also shows structural homology with the extracellular domains of TF (25, 26) and ligand bound IFNγR (27), and even a natural killer cell inhibitory receptor (28). Tissue factor and IFNγR are “class 2” cytokine receptors (14) in which the seven-ß-strand FNIII-like fold is maintained but the relative orientation of the two domains differs.

The interaction between EMP1 and EPOR consists of two molecules of EMP1 bound by two molecules of EPOR to form a two-fold symmetrical assembly, where each peptide interacts with its peptide partner and both receptor molecules (17). As a result of this binding symmetry, EPOR residues that interact with EMP1 are practically identical for each EPOR in the complex. These interactions include hydrophobic contacts with the side chains of Phe93, Met150, and Phe205 and a hydrogen-bonding interaction with the side chain of Thr151 of the EPOR. The interactions with Phe93, Met150, and Phe205 are among the most significant non-polar contacts in the binding interface in terms of the area of molecular surface buried by each of these residues (17). Prior to the determination of the EMP1-EPOR crystal structure, we investigated what role Phe93 and Met150 might have in EPO binding, based on their predicted secondary structural homologies to the positions of ligand binding determinants in other receptors of the cytokine receptor family (29). Phe93 was found to be critical for EPO binding, while Met150 was found to be relatively unimportant. In the present study, we used the alanine substitution mutants at Phe93 and Met150 to determine if these residues contribute significantly to EMP1 binding. In addition, further alanine substitution mutants were created at EMP1 residues Thr151 and Phe205. These residues were selected for mutagenesis, based on their contacts with EMP1 in the EMP1-EPOR crystal structure and on predicted secondary structural homologies to the positions of ligand binding determinants in other receptors of the cytokine receptor family. Furthermore, these residues contact EPO in the recently reported EPO-EPObp crystal structure (20). Our results indicate that Phe93 and Phe205 are important for both EPO and EMP1 binding, while Thr151 appears to be unimportant for the binding of these ligands. In contrast, Met150, which is relatively unimportant for EPO binding, is critical for EMP1 binding. Thus, some of the same residues on the EPOR are important for binding two very different ligands, EPO and EMP1, suggesting that these residues may be required for productive ligand binding to the EPOR.

EXPERIMENTAL PROCEDURES

Expression and Mutagenesis of the EPOR—The bacterial expression, purification, and characterization of the 225-amino acid extracellular domain of the EPOR (referred to here as EBP) has been described in detail elsewhere (16). Biophysical analysis indicated that the purified EBP contained the expected amino terminus, disulfide-bridging pattern, and molecular mass. The EBP exhibited a low nanomolar binding affinity (Kd = 5 nM) for EPO. Mutagenesis of the EBP was performed as described previously (29), and the concentrations of purified wild-type and mutant EBP were estimated using the experimentally determined extinction coefficient of the wild-type EBP at 2.3 absorbance units/mg/ml at 280 nm (16). Gross structural characterization of the F205A-EBP by circular dichroism (CD) was performed as described previously (29).

Assay of the Binding Activity of Wild-type and Mutant EBP—Competition binding assays in which wild-type or mutant EBP compete for 125I-EPO binding with EPOR on the surface of TF-1 cells were performed as described (30). Equilibrium binding experiments on the wild-type and mutant EBP were carried out using a surface plasmon resonance assay on a BIAcore 2000 instrument. Proteins were coupled to the carboxymethylated dextran surface using amine coupling chemistry. The derivatized dextran was immobilized with 3,000 resonance units. Different concentrations were estimated in each flow cell sequentially at a flow rate of 5 µl/min. The surfaces were regenerated with a 1-min pulse of 10 mM sodium acetate, pH 4.0. Each chip had one negative control surface and one surface containing wild-type EBP. To calculate the dissociation constants, the change in the equilibrium amount of ligand bound as a function of the concentration of ligand was fit to the equation for a simple 1:1 binding model,

\[ R = \frac{R_{\text{max}} \times [\text{ligand}]}{K_D + [\text{ligand}]} + R_{\text{max}} \]

or to a model for two independent binding sites

\[ R = \frac{R_{\text{max1}} \times [\text{ligand}]}{K_{D1} + [\text{ligand}]} + \frac{R_{\text{max2}} \times [\text{ligand}]}{K_{D2} + [\text{ligand}]} \]

where R is the response, R_{max} is the maximum response, and K_D is the dissociation constant.

**EPO and EMP1 Binding Determinants of the EPO Receptor**

Expression and Mutagenesis of the EBP—The ability of EBP to mediate the dimerization of wild-type and mutant EBP was evaluated in chemical cross-linking assays using the sulphydryl-reactive cross-linking reagent (1,4-di-(3’-2’-pyridyldithio) propionamidobutane (DPDPB; Pierce). The assay was performed as described previously (17, 30), except that the reaction products were detected and quantified using high performance-size exclusion chromatography (HP-SEC). Briefly, wild-type or mutant EBP (11 µM) were incubated with a variable concentration of EMP1 (stock prepared in 0.1% trifluoroacetic acid) and 0.5 mM DPDPB (stock prepared in dimethyl sulfoxide) in 75 µl of phosphate-buffered saline, pH 7.4, for 4 h at room temperature then overnight at 4 °C. All reactions and controls contained a final concentration of 4.4% Me2SO and 0.007% trifluoroacetic acid to improve the solubility of the cross-linker. The samples were analyzed by HP-SEC on a Waters 625 HPLC system equipped with a Waters 996 detector. Separations were performed at room temperature on a 7.8 × 300-mm G3000 SWXL column (Supelec, Bellfonte, PA). The column was equilibrated in 10 mM Na2PO4, pH 7.2, 150 mM NaCl at a flow rate of 1 ml/min and was monitored at 220 nm. Under these conditions, EBP eluted at ~9.6 min, while the dimer product eluted earlier at ~8.9 min. These elution times correspond to M, ~25,000 for EBP monomer and M, ~52,000 for the dimer product based on calibration of the column using a commercial HP-SEC standard mixture (16). The percentage of dimer product formed was calculated by adding the integrator determined peak area for the monomer and dimer protein peaks to determine total protein peak area. The dimer area value was divided by the total protein peak area and multiplied by 100 to yield the percentage of dimer observed in each reaction mixture. The reported value for each concentration is the average over three different chromatographic separations as well as the error bars are the standard deviation value for the three experiments.

**STAT Activation Assay**—For expression in COS7 cells, the various mutants were subcloned from the EBP bacterial expression plasmid pSAM3 (16) to a mammalian expression plasmid encoding the full-length human EPOR in pSG5 (Stratagene, La Jolla, CA). Transient transfection of COS7 cells (ATCC, Rockville, MD) was performed using LipofectAMINE (Life Technologies, Inc.) per the manufacturer’s instructions. Cells were transfected with expression plasmids encoding the wild-type or mutant (P93A, M150A, or F205A) forms of the human EPOR along with a plasmid encoding STAT5a (pME185-STAT5a, kindly provided by Dr. Alice Mui, DNAX Research Institute). Stimulation experiments were performed for 10 min at 37 °C with either EPO or EMP1 at the stated concentrations. Electrophoretic mobility shift assays were performed using nuclear extracts prepared from resting or stimulated cells and an oligonucleotide probe containing the FcγRI STAT response sequence, as described previously (31).

Previously, we expressed the extracellular domain of the human EPOR as a soluble EBP in E. coli (16). The crystal structure of the EBP bound to an EPO-mimetic peptide (EMP1) revealed several EBP residues that form significant contacts with the peptide (17). These include major non-polar interactions with the side chains of Phe93, Met150, and Phe205 and a hydrogen bond with the side-chain hydroxyl group of Thr151.
concentrations. About 45% inhibition of $^{125}$I-EPO binding to EBP does not compete for EPO in this assay except at very high
values of $^{125}$I-EPO binding (Fig. 2). In contrast, the F205A-EBP interacts with Tyr P4, Phe P8, Trp P13, and Cys P15 of the peptide to form the hydrophobic core of the interaction between EBP and EMP1. The hydrogen bond with the hydroxy group of Thr151 is part of a network of mostly main-chain hydrogen bonds formed with main-chain atoms of the type I $\beta$-turn of the peptide. The six loops containing binding determinants in receptors or the cytokine receptor family are labeled L1-L6. For clarity, the side chains of Phe P8 and Cys P15 are not shown.

To determine if Thr151 and Phe205 contribute significantly to EPO binding, these residues were replaced with alanine and the resultant mutant proteins were purified and tested for their ability to compete for $^{125}$I-EPO binding with EPOR on the surface of TF-1 cells. Alanine was chosen as the replacement residue, since it is likely to result in a loss of interactions from the original side chain without introducing new interactions or gross structural changes (32, 33). The T151A-EBP exhibited an IC$_{50}$ of 6.5 nM in this assay, nearly identical to the wild-type value (Ref. 17; Fig. 1). The hydrogen bond with the hydroxy group of Thr151 is part of a network of mostly main-chain hydrogen bonds formed with main-chain atoms of the type I $\beta$-turn of the peptide (Fig. 1).

F205A-EBP produced a CD spectrum similar to wild type except for reduced intensity in the 200–220-nm region (Fig. 3A) and resulted in a small ($\sim5 ^\circ$C) decrease in thermal stability relative to the wild-type EBP (Fig. 3B). Taken together, the limited differences in CD spectrum and decrease in $T_m$ of the F205A-EBP indicate that this mutation does not cause significant changes in the global secondary structure or the stability of the EBP.

To determine an EPO binding affinity ($K_d$) for the EBP mutants, equilibrium binding analyses were performed on the wild-type and mutant EBP using surface plasmon resonance. As can be seen in Fig. 4A, the EPO binding curves for the M150A- and T151A-EBP were similar to wild type. The data for the wild-type and T151A-EBP fit best to a two-site model (see “Experimental Procedures”), yielding a high affinity $K_d$ of $\sim5$ nm and a low affinity $K_d$ of $\sim900$ nm for EPO. For the M150A-EBP, however, the two-site fit of the data gave no improvement over the 1:1 model. This mutant exhibited a single $K_d$ for EPO of 36 nm, only slightly increased relative to the high affinity (5
exhibiting a
thermore, in the competition binding format, the EBP mutants
response was not high enough above controls to be attributed
to EPO over the concentration range studied (1 nM to 10
EPO binding determinant. The F93A-EBP showed no binding
the CD spectrum of the EBP (29). Thermal denaturation curves
were generated by plotting the ellipticity at 228 nm derived from
the CD spectra at each temperature using 0.365 mg/ml protein in phos-
phate-buffered saline. The F205A mutation causes a small decrease in
the CD spectrum at 200 to 220 nm. The F93A mutation results in essentially no change in
CD spectra (29). Thermal denaturation curves (200 to 220 nm. The F93A mutation results in essentially no change in
phosphate buffered saline. Overall, the CD spectrum of the F205A-EBP
A CD spectra (average and standard error, respectively, of three independent experi-
ments). The dissociation constants (Kd) for EPO derived from these data are 5 nM (high affinity) and 900 nM (low affinity) for both the wild-type and T151A-EBP, and 36 nM (single affinity) for the M150A-EBP. The Kd
for the F205A-EBP is 1
M and the F93A-EBP did not show detectable EPO binding in this assay, indicating that Phe93 and Phe56 are important EPO binding determinants. B, sensograms showing the response of the wild-type and mutant EBP to 1 μM EMP1. The T151-EBP bound EMP1 about as well as the wild type. The M150A-, F93A-, and F205A-EBP did not show any binding to EMP1 at this concentration, exhibiting responses similar to the background observed for a blank surface or surfaces coated with the unrelated proteins leptin and leptin receptor.

with certainty to specific binding (data not shown). Therefore, if the peptide binds at all to these mutants, it is with a much lower affinity (>10 μM) than for wild type (0.35 μM). These data indicate that Phe93, Phe205, and Met150 are all critical binding determinants for EMP1.

The ability of EPO and EMP1 to bind and signal through full-length wild-type, F93A-, M150A-, and F205A-EBP was tested in a STAT activation assay. The T151A mutation was not tested in this assay, since this mutation did not appear to have a significant effect on either EPO or EMP1 binding. COS
cells cotransfected with full-length receptor and STAT5a con-
structs were treated with EPO or EMP1, and active STAT5a
sensitive element was detected in gel shift assays. Initial experi-
ments were performed with concentrations of ligand that elic-
ited a good response for the wild-type EPO, but none of the
mutants showed a response to EMP1 (Fig. 5A). A faint band
was observed for the wild-type EPO at 0.24 nM EPO, but no band
detectable at this concentration for the M150A-EBP, and active STAT5a
dimer bound to an oligonucleotide containing a STAT-responsive
element was detected in gel shift assays. These data indicate that Phe93, Phe205, and Met150 are all critical binding determinants for EMP1.

Surface plasmon resonance was also used to examine EMP1
binding affinities of the wild-type and mutant EBP. The sen-
sograms for EMP1 binding at a concentration of 1 μM are shown in Fig. 4B. The F93A-, M150A-, and F205A-EBP did not show specific binding to EMP1 above the background seen for an undervatized surface or surfaces coated with unrelated proteins (leptin and leptin receptor). Only the T151A-EBP
bound EMP1, showing a response similar to that of wild type
(Fig. 4B). In binding curves generated at various concentrations of
EMP1 (0.1–10 μM), the wild-type and T151A-EBP had comparable affinities for EMP1 of ~350 nM (data not shown).

This value is similar to the half-maximal response (EC50) of
400 nM reported for EMP1 in cell proliferation assays (19). The
F93A-, M150A-, and F205A-EBP showed slight binding only at
μM concentrations of EMP1 (from 6 to 10 μM); however, the
response was not high enough above controls to be attributed
to EMP1. The F93A-EBP, however, exhibited the opposite pattern. It still did not respond to EPO but
demonstrated a significant response to EMP1. Finally, the M150A-
EBP did not show a response to EPO or EMP1 at the high

FIG. 3. CD spectrum and thermal stability of the F205A-EBP. CD spectra (A) were generated at 25 °C and 0.365 mg/ml protein in phosphate-buffered saline. Overall, the CD spectrum of the F205A-EBP is similar to that of the wild-type EBP except for reduced intensity from 200 to 220 nm. The F93A mutation results in essentially no change in the CD spectrum of the EBP (29). Thermal denaturation curves (B) were generated by plotting the ellipticity at 228 nm derived from the CD spectra at each temperature using 0.365 mg/ml protein in phosphate-buffered saline. The F205A mutation causes a small decrease in the Tm of the EBP. The Tm of the F93A-EBP is identical to that of the wild-type EBP (29).

FIG. 4. Analysis of EPO and EMP1 binding to the wild-type and mutant EBP by surface plasmon resonance. A, equilibrium bind-
ing analyses of EPO binding to the wild-type and mutant EBP. The
lines are the best fit of the data to Equations 1 or 2 (see “Experimental Procedures” and “Results”), and the symbols and vertical bars are the average and standard error, respectively, of three independent experi-
ments. The dissociation constants (Kd) for EPO derived from these data are 5 nM (high affinity) and 900 nM (low affinity) for both the wild-type and T151A-EBP, and 36 nM (single affinity) for the M150A-EBP. The Kd
for the F205A-EBP is 1 μM and the F93A-EBP did not show detectable EPO binding in this assay, indicating that Phe93 and Phe56 are important EPO binding determinants. B, sensograms showing the response of the wild-type and mutant EBP to 1 μM EMP1. The T151-EBP bound EMP1 about as well as the wild type. The M150A-, F93A-, and F205A-EBP did not show any binding to EMP1 at this concentration, exhibiting responses similar to the background observed for a blank surface or surfaces coated with the unrelated proteins leptin and leptin receptor.
concentrations. The loss of a response to EPO at 1.23 μM for the M150A-EPOR is probably due to supersaturating concentrations of ligand occupying the receptor binding site at a 1:1 ratio, thereby driving the receptor to monomerization and reducing signaling. This effect was seen for the wild-type EPOR at 1.23 μM EPO and at 20 and 200 μM EMP1 (Fig. 5, A and B). Evidently, the M150A-EPOR binds EPO well enough to exhibit this effect. To be sure that supersaturating concentrations of ligand were not inhibiting the signaling of the other mutants, intermediate concentrations of 49 nM EPO and 100 μM EMP1 were tested. Under these conditions, none of the mutants showed a response to EPO and only the F93A-EPOR showed a response to EMP1, which was less than that observed at 200 μM EMP1 (data not shown).

To investigate further the effects of the EBP mutations on the interaction with peptide, an EBP cross-linking assay was used to evaluate the ability of EMP1 to mediate the dimerization of the EBP mutants (17, 30). Wild-type and mutant EBP were incubated with various concentrations of peptide and then cross-linked and analyzed by HP-SEC. The percentage of dimer formed was calculated from the areas under the peaks and plotted for each concentration of peptide. The symbols and vertical bars are the average and standard error, respectively, of three independent chromatographic separations.

These data indicate that the F93A mutation and especially the M150A mutation result in a large decrease in the ability of EMP1 to facilitate the dimerization of the EBP. Taken together with the results of the equilibrium binding and STAT activation assays, these results indicate that Phe93, Phe205, and Met150 are all important for the binding and/or agonist activity of EMP1.

**DISCUSSION**

In this study, we investigated the effects of alanine substitution of EPOR residues Phe93, Phe205, Met150, and Thr151 on binding to both EPO and an EPO-mimetic peptide (EMP1). These residues were chosen for investigation based on several criteria. First, all of these residues interact with EMP1 in the crystal structure of EPOR bound to the EBP (17), suggesting that they may contribute significantly to EMP1 binding. Second, Phe93 has been shown to be critical for EPO binding (29), suggesting that residues that interact with peptide in the EMP1-EBP crystal structure might also be involved in EPO binding. Third, many cytokine receptors contain ligand binding determinants in positions homologous to Phe93, Met150, and Thr151 (29) and Phe205 (35–42), further suggesting that these residues may be involved in ligand binding to the EPOR. In addition, all these residues are buried in the interface with EPO in the EPO-EPOR crystal structure (20).

Although both Phe93 and Phe205 are important binding determinants for EPO, their relative contributions to the binding of this ligand differ. For example, the F205A-EPOR exhibits slight activity at 1.23 μM EPO in the STAT activation assay, while the F93A-EPOR does not. The activity seen for the F205A-EPOR at 1.23 μM EPO might be expected, based on the 1 μM K_d for EPO of the F205A-EBP in the equilibrium binding assay. In the same vein, the F93A-EPOR might not be expected to respond to 1.23 μM EPO in the STAT activation assay, since the F93A-EBP did not exhibit detectable binding to concentrations of EPO as high as 10 μM in the equilibrium binding assay. It is unlikely that the lack of activity of the F93A-EPOR in the STAT assay results from poor expression on the surface of the COS cells used in the assay, since this mutant exhibited expression on COS cells comparable to wild type (29). Combined, these results suggest that Phe93 is more important for EPO binding than Phe205. The 200-fold increased K_d for EPO of the F205A-EBP compared with the 1,000-fold increased IC50 for EPO reported for the F93A-EBP (29) adds further support to this hypothesis.

The data presented here indicate that Phe93, Phe205, and Met150 are all important binding determinants for EMP1, since none of the respective mutant proteins exhibit detectable bind-
ing to concentrations of EMP1 up to 10 μM in the equilibrium binding assay, while Thr151 is relatively unimportant for EMP1 binding in this assay. In addition, the F205A- and M150A-EPOR do not respond to peptide in the STAT activation assay, while the F93A-EPOR did show a response, but only at high concentrations of EMP1 (100 and 200 μM). These effects could be the result of the reduced binding of EMP1 or aberrant dimerization and/or signaling of the mutant receptors. In any case, the results of the STAT activation assay indicate that Phe93 is not as important as Phe205 and Met150 for activity with EMP1. The results of the EMP1-mediated dimerization (cross-linking) assay suggest that Phe93 and especially Met150 are important for the dimerization of the EBP. Phe205 appears to be relatively less important for dimerization, since the F205A-EBP exhibits significant dimerization in the presence of peptide, albeit at higher concentrations. Apparently, the ability of EMP1 to mediate the dimerization of the F205A-EBP does not translate to activity in the STAT assay. The presence of the cross-linker capturing a transient dimerization of this mutant at the high concentrations of EMP1 used in the cross-linking assay might explain this discrepancy. The relatively small effect of the F205A mutation in the cross-linking assay does not mean that Phe205 is unimportant for EMP1 binding, since the amount of dimerization observed for the EBP mutants may not be related to relative binding affinities due to the high concentrations of protein used in the assay. In addition, the presence of the cross-linking reagent complicates interpretation of the results, since different modes of binding and/or dimerization resulting from the mutations may affect the cross-linking reaction.

Of the 45 amino acid residues (20%) of the EBP that we have examined by alanine substitution to date (29, 43), only Phe93 and Phe205 were found to be critical for EBP binding. The remaining residues had relatively little or no role in EBP binding or were important for structure. These results are consistent with the suggestion that only a few residues make significant contributions to binding in protein-protein interactions (functional epitope), even though a large number of residues may be involved in the contact interface (structural epitope; Refs. 44–46). In the GH-GHR interaction, two trytophans (Trp104 and Trp169) accounted for the majority of the binding free energy of the interaction, constituting a hot spot for GH binding to GHR (44). Alaniine substitution of Trp104 and Trp169 resulted in the largest decreases in binding affinity by far, estimated to be greater than 2,500-fold, relative to wild-type GHbp (44, 47). Our results suggest that Phe93 and Phe205 of the EPOR may be functionally analogous to Trp104 and Trp169 of the GHR in providing the majority of the binding energy for the interaction with ligand. When the EPO-EPObp crystal structure became available (29), we found we had already examined the function of 40% of the residues involved in H-bonds or salt bridges and nearly 50% of the residues involved in non-polar interactions with EPO (29, 43). The data presented here, combined with the fact that Phe93 and Phe205 dominate the non-polar contacts with EPO site 1 and site 2 (20), indicate that these residues are an important component of the hot spot for EPO binding.

In the EPO-EPObp crystal structure, Met150 contacts only a single residue (Phe93) in binding site 1 on EPO and is buried in site 2, contacting three EPO residues (20). Our results indicate that the single contact with site 1 is unimportant for EPO binding and the more extensive contacts with site 2 are apparently also relatively unimportant for EPO binding, since the response of the M150A-EPOR to EPO in the STAT activation assay was similar to that of wild type. In the case of EMP1 binding to the EPOR, Met150 buries significant surface area with EMP1 (~60 Å2; Ref. 17) and is critical for EMP1 binding. However, Thr151 also forms hydrophobic interactions with peptide in the EMP1-EBP crystal structure. In addition, the side-chain hydroxyl of Thr151 forms a hydrogen bond with peptide (see Fig. 1). Despite these interactions, Thr151 does not contribute significantly to EMP1 binding. Apparently, the deletion of a single hydrogen bond in the network of hydrogen bonds with peptide in the region around Thr151 (see Fig. 1) is not sufficient to disrupt peptide binding. These results support the conclusion that the amount of surface area buried by a contact residue does not always correlate with binding affinity (44–46) and emphasize the importance of mutagenesis studies to verify structural based predictions of the function of specific residues.

In conclusion, the fact that residues important for binding EPO (Phe93 and Phe205) are also important for binding the unrelated peptide, EMP1, suggests that these residues may represent a minimum epitope for productive ligand binding to the EPOR and has important implications for the design of small molecule mimetics. In addition, our results suggest that, as is the case for GHR, only a few residues of the EPO contribute significantly to ligand binding (Phe93 and Phe205 for EPO binding and Phe93, Phe205, and Met150 for EMP1 binding). Thus, our results provide further support for the notion that the functional epitope for ligand binding is relatively small compared with the structural epitope. Indeed, this may account for the existence of a relatively small peptide mimetic of EPO (EMP1) in the first place and indicates that it may be possible to discover non-peptide small molecule mimetics of EPO and other biologically important cytokines.

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