Identification of a 13 Amino Acid Peptide Mimetic of Erythropoietin and Description of Amino Acids Critical for the Mimetic Activity of EMP1[†]

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ABSTRACT: To obtain information about the functional importance of amino acids required for effective erythropoietin (EPO) mimetic action, the conserved residues of a peptide mimetic of EPO, recently discovered by phage display, were subjected to an alanine replacement strategy. Further, to identify a minimal mimetic peptide sequence, a series of truncation peptides has been generated. One EPO mimetic peptide sequence, EMP1, was targeted and more than 25 derivatives of this sequence were evaluated for their ability to compete with [^{125}I]EPO for receptor binding and for their ability to support the proliferation of two EPO-responsive cell lines. Two hydrophobic amino acids, Tyr^4 and Trp^{13}, appear essential for mimetic action, and aromatic residues appear to be important at these sites. These findings are consistent with the previously reported X-ray crystal structure of EMP1 complexed with the extracellular domain of the EPO receptor (EPO binding protein; EBP). In our efforts to define the structural elements required for EPO mimetic action, a 13 amino acid peptide was identified which possesses mimetic properties and contains a minimal agonist epitope. The ability of this peptide to effectively serve as a mimetic capable of the induction of EPO-responsive cell proliferation appears to reside within a single residue, equivalent to position Tyr^4 of EMP1, when present in a sequence that includes the cyclic core peptide structure. Although these peptides are less potent than EPO, they should serve as an excellent starting point for the design of compounds with EPO mimetic activity.

Erythropoietin (EPO)[1] is a 34 kDa glycoprotein which regulates the production of red blood cells (*1, 2*). The actions of this hormone are mediated, in part, by interaction with a type I-cytokine family receptor which initiates a cascade of intracellular events leading to proliferation and differentiation of EPO-responsive cells (*3, 4, 5*). We have recently described a series of agonist peptides capable of supporting erythropoiesis in a manner similar to EPO (*6*) and reported the X-ray crystal structure of a representative member of this series (EMP1) complexed with the extracellular ligand binding domain of the erythropoietin receptor (erythropoietin binding protein; EBP) (*7*). The sequences of these EPO mimetic peptides are unrelated to the primary sequence of EPO and their ∼2 kDa molecular weight makes them significantly smaller than the natural ligand. A significant feature of the peptide−receptor interaction is the ability of the mimetic peptide to promote both the crystallographic and solution phase dimerization of the EBP (*7*). EBP dimerization is mediated by the noncovalent homodimeric association of two peptides, with residues from each monomer of the peptide dimer making contact with both receptors. Thus, the peptide dimeric assembly is a key component in both receptor binding and dimerization.

The set of EPO mimetic peptides (20 amino acids; detailed in Figure 2 of ref *6*) is characterized by a conserved motif of xxxYxCxxGPxTWxCxPxxx, in which the cysteine residues are oxidized to form an intramolecular disulfide bond and the indicated residues appear exclusively or at high frequency. Structurally, the GPxTW region forms a slightly distorted type 1 β-turn and is primarily involved in peptide−receptor contacts. The Tyr^4 side-chain hydroxyl makes the only side-chain hydrogen-bond interaction from the peptide

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[1] Abbreviations: erythropoietin, EPO; erythropoietin binding protein, EBP; growth hormone; GH; *tert*-butoxycarbonyl, BOC; trifluoroacetic acid, TFA; hydrofluoric acid, HF; acetic acid, HOAc; acetonitrile, ACN; dimethyl sulfoxide, DMSO, phosphate buffered saline, PBS; bovine serum albumin; BSA; [(1,4-di-[3′-(2′-pyridyldithio)propionamido]butane, DPDPB. All peptide positions are numbered sequentially from the *N*-terminus relative to the EMP1 sequence using three-letter or one-letter amino acid code and a superscript number, i.e., Tyr^4, regardless of their length.

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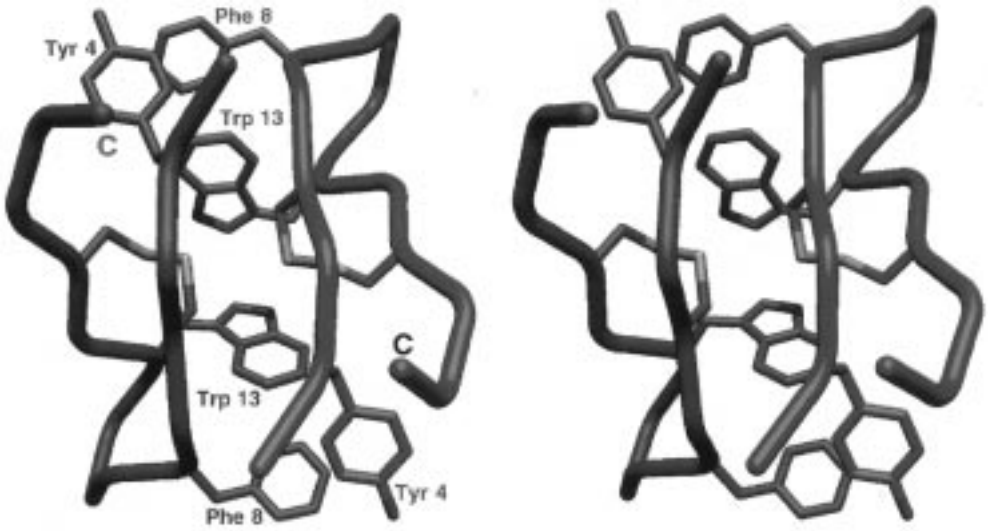

FIGURE 1: Stereoview of the two EMP1 peptides from the EBP-EMP1 crystal structure. Illustrated is the intricate hydrophobic core of the peptide−peptide interaction mediated by close interaction between the two Trp^{13} residues, and an essential contribution from the disulfide bridges and with Phe^{8} and Tyr^{4} crossing over from the other peptide partner in a symmetrical fashion. All are conserved residues in the family of phage detected EPO receptor binding sequences except Phe^{8}. The hydrophobic peptide−peptide core provides the framework for the hydrophobic interaction with the two receptor molecules in the crystal structure with each EBP molecule having close hydrophobic interactions with Phe^{8}, Trp^{13} from one peptide and Tyr^{4} from the other.

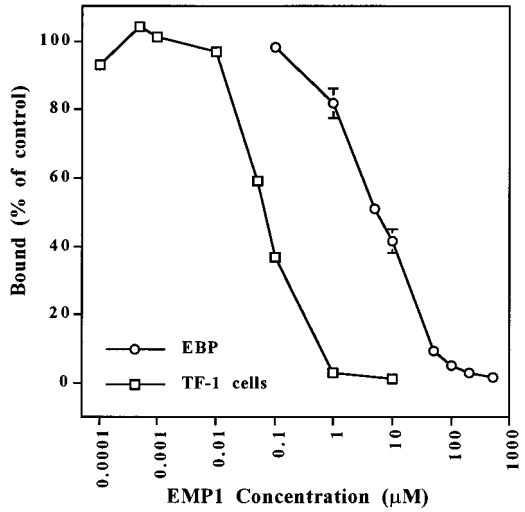

FIGURE 2: Competitive binding activity of EMP1 in two different assay formats. [^{125}I]EPO (0.5 nM) was added to EPO binding protein immobilized on agarose beads or TF-1 cells and increasing concentrations of EMP1 added as indicated. The EMP1 IC_{50} value was determined to be 5 ± 2 μM on EBP and 0.07 ± 0.02 μM on cells. This difference in competitive binding behavior appears to be related to the potential of TF-1 cells to allow free receptor dimerization. The EPO mimetic peptides reported here were subjected to a similar analysis and their competitive binding ability reported in Tables 1−4.

to the receptor (*7*). The peptide regions around the cysteines form a short β-sheet hairpin structure that is stabilized by a complex array of intramolecular and intermolecular interactions. This intricate assembly of peptide amino acids forms a core in which hydrophobic residues including the disulfide bridge, Tyr^{4}, Phe^{8}, and Trp^{13} interact to create a hydrophobic framework for receptor interaction (Figure 1). Among these residues only Phe^{8} is not conserved in the family of phage selected sequences (*6*).

To explore more fully the structural features which contribute to the ability of an EPO mimetic peptide to compete for EPO binding to the receptor and to support the proliferation of EPO-responsive cells, we investigated the role played by conserved residues of the peptide family. A set of peptides, derivative of a single EPO-mimetic peptide sequence, was generated by alanine replacement of conserved residues. Additionally, to determine the minimal unit required for an effective EPO receptor agonist, a truncation series was synthesized. Here, we report these studies, with supporting data from peptides synthesized to confirm some of these observations, and then correlate these sequence−activity studies with the EBP-EMP1 X-ray crystal structure.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Generically, all of the cyclic EPO mimetic peptides were synthesized using Merrifield solid-phase peptide synthesis methodology on Applied Biosytems Models 430A and 431A Peptide Synthesizers. Briefly, protected BOC (*tert*-butoxycarbonyl) amino acid derivatives were coupled as hydroxylbenzotriazole esters after acid deprotection [50% trifluoroacetic acid (TFA)/50% dichloromethane] and neutralization (5% diisopropylethylamine/*N*-methylpyrrolidone) steps. The acid deprotection, neutralization, and coupling steps were repeated until the full-length peptide-resin was obtained. The completed resin-bound peptide was treated with 50% TFA to yield the free *N*-terminal peptide-resin. All peptides were amidated at the C-terminus. The side-chain protecting groups were removed and the peptide was cleaved from the resin by treatment with liquid HF-anisole (10 vol %) for 90 min at −6 °C. The free peptide was then precipitated with cold ethyl ether. Additional ether washes were employed to remove residual scavenger (anisole). The dried peptide-resin was then extracted in 50% HOAc, diluted with water, and lyophilized. The cysteines were oxidized by suspending the crude peptide in 0.1% TFA/acetonitrile followed by dilution with water to a peptide concentration of 1 mg/mL, which yielded a clear solution (typically 30−50% AcN/water). Solid ammonium bicarbonate was added to the peptide solution until a pH of ca. 8.0 was obtained. Potassium ferricyanide (0.1 M) was

added dropwise to the stirred peptide solution until a light yellow solution persisted. This peptide solution was monitored by analytical reverse-phase HPLC for the formation of oxidized product. The cyclic peptides typically eluted after the reduced starting material on a Vydac C18 column. The cyclization reaction time varied from 20 min to 18 h at RT, depending on the particular peptide sequence. Before further purification, Bio-Rad AG 2-X8 anion-exchange resin (chloride form) was added to the yellow peptide solution to remove the oxidizing agent (iron). The anion-exchange resin was removed by filtration and washed with a volume of water equal to the volume of the peptide solution. The combined filtrates were loaded onto a preparative HPLC column (Vydac C18) and the target peptide was isolated at 90−95% purity using a water-acetonitrile gradient with 0.1% TFA. If significant linear peptide remained after the initial chromatographic procedure, the oxidation step with potassium ferricyanide was repeated to maximize the overall yield of the oxidized peptide. The high-purity peptide fractions collected from the preparative chromatography protocol were pooled and lyophilized. The final product of each synthesis was characterized by analytical HPLC for purity (usually >95%), ion-spray mass spectroscopy for molecular weight and amino acid analysis for peptide content. All cyclic peptide preparations reported here were negative for free sulfhydryl groups using Ellman's Reagent (*8, 9*). EMP39 was synthesized by Quality Controlled Biochemicals (Hopkinton, MA) using a differential side-chain protection strategy for the formation of an amide bond between the E6 and K15 side chains.

EPO Competition Binding Assay. The extracellular domain of the human erythropoietin receptor, EBP, was produced, characterized, and immobilized as described (*10*). EBP produced by this method contains one free sulfhydryl group which can be modified without affecting the solution phase binding of ligand. This observation was extended to produce immobilized EBP, as the basis for a competition binding assay, by the covalent attachment of EBP to Sulfolink agarose beads (Pierce Chemical Co). Briefly, individual peptides were dissolved in DMSO to prepare a stock solution at 1 mM. All reaction tubes (in duplicate) contained 50 μL of EBP beads, 0.5 nM [^{125}I]EPO and 0−500 μM peptide in a total volume of 500 μL binding buffer (PBS/0.2% BSA). The final concentration of DMSO was adjusted to 2.5% in all peptide assay tubes. Concentrations of up to 25% DMSO (vol/vol) were shown to have no deleterious effect on binding (data not shown). Nonspecific binding was measured in each assay by inclusion of tubes containing a large excess of unlabeled EPO (1000 nM). Initial assay points with no added peptide were included in each assay to determine total binding. Binding mixtures were incubated overnight at room temperature with gentle rocking. The beads were then collected using Micro-columns (Isolab, Inc.) and washed with 3 mL of wash buffer (PBS/5% BSA). The columns containing the washed beads were placed in 12 × 75 mm glass tubes and bound radioactivity levels determined in a γ counter. The amount of bound [^{125}I]EPO was expressed as a percentage of the control binding (total = 100%) and plotted versus the peptide concentration after correction for nonspecific binding. The IC_{50} was defined as the concentration of the analyte which reduced the binding of [^{125}I]EPO to the EBP beads by 50%. At least two independent determinations were performed on each peptide variant.

Erythropoietin Receptor Competition Binding Analysis. TF-1 cells were maintained in RPMI 1640, 10% fetal calf serum, 1% L-glutamine, 1% penicillin, 0.1% streptomycin, and 1 ng/mL of GM-CSF. [^{125}I]EPO was obtained from NEN Research Products. Cells were centrifuged and washed 1 time with binding buffer (RPMI 1640, 5% BSA, 25 mM Hepes, pH 7.5, 0.02% sodium azide) resuspended in binding buffer and counted using trypan blue as an indicator of viability. Each reaction contained approximately 5×10^5 cells, [^{125}I]EPO (0.5 nM), no competitor or peptide in a final volume of 200 μL. The binding reactions (in triplicate) were incubated overnight at 4 °C. Following binding, the tubes were centrifuged at 12 000 rpm for 2 min at 4 °C in a refrigerated centrifuge. The supernatant was removed, the cell pellet resuspended in 100 μL of binding buffer, and the cell suspension layered onto 0.7 mL of bovine calf serum. The tubes were centrifuged at 12 000 rpm for 5 min at 4 °C, the supernatant was removed, the bottom of the tubes snipped off, and the cell pellets counted in a Micromedic ME plus γ counter. Nonspecific binding was determined by incubating cells with [^{125}I]EPO and a 100-fold excess of nonradioactive EPO.

EPO-Dependent Cell Proliferation Assays. Cell lines FDC-P1/tHER and FDC-P1/HER expressing a functional, truncated human EPO receptor (missing the C-terminal 40 amino acids) and native human EPO receptor (*6*), respectively, were grown and maintained as described previously (*11*). Both cell lines exhibit EPO-dependent cellular proliferation. Briefly, cells were maintained in RPMI 1640 media (Gibco/BRL) containing 10% heat-inactivated fetal calf serum and 1 unit/mL of recombinant human EPO. For the cellular proliferation assay, cells were grown to stationary phase, centrifuged, washed with RPMI 1640 media (no EPO), and incubated in medium without EPO for 18 h.

At 18 h, the cells were counted and dispensed at 40 000 cells/well. Stock solutions of the respective peptides (10 mM in DMSO) were prepared and dispensed in triplicate to final concentrations of 0.1 nM through 10 μM in a final volume of 0.2 mL. Final DMSO concentrations of 0.1% (vol/vol, maximal) or less were shown to have no cellular toxicity or stimulatory effects (data not shown). A standard EPO dose response curve was generated with each assay series. After a 42 h incubation at 37 °C (ca. 2 cell doublings), 1 μCi/well of [^{3}H]thymidine was added, and the incubation continued for 6 h, at which time the cells were harvested and counted to assess [^{3}H]thymidine incorporation as a measure of cell proliferation. Results are expressed as the amount of peptide necessary to yield half of the maximal activity obtained with recombinant EPO.

Dimerization of EBP by EPO Mimetic Peptides. Crosslinking was used to study the ability of EPO mimetic peptides to mediate the dimerization of the ligand binding domain of the EPO receptor and has been described (*7*). Peptide-mediated dimerization was stabilized for study using the nonwater soluble homobifunctional sulfhydryl-reactive crosslinking reagent DPDPB [(1,4-di-[3′-(2′-pyridyldithio)propionamido]butane], Pierce Chemical Co. Briefly, EBP (22 μM) was incubated in the presence or absence of DPDPB (1.1 mM) and 400 μM of peptide in 75 μL of PBS, pH 7.5, with all reactions and controls containing a final concentra-

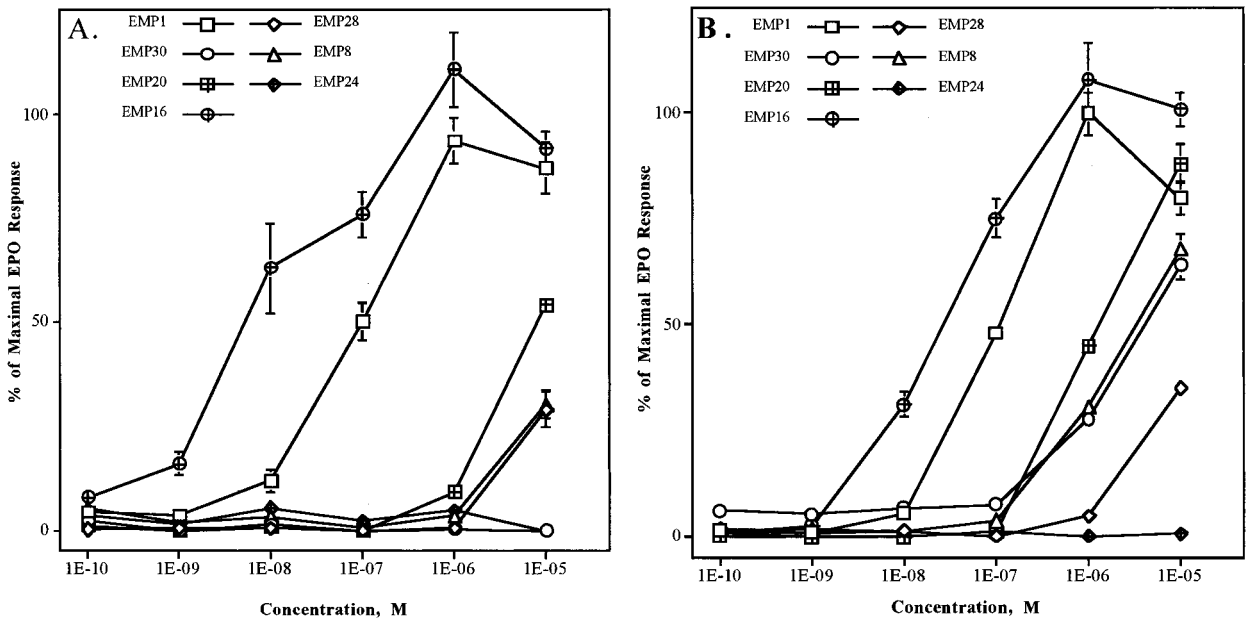

FIGURE 3: EPO responsive cell line proliferation. EPO responsive cells [panel A, FDC-P1/HER cells expressing the full-length EPO receptor; panel B, FDC-P1/trHER expressing the truncated receptor (see text for details)] were incubated with increasing concentrations of EMP1 and the resultant cell proliferation determined by [^{3}H]thymidine incorporation. In each experiment, an EPO proliferation curve was performed and a 50% maximal value determined. The concentration of EMP1 which resulted in a 50% maximal level of radiolabel incorporation was reported as the ED_{50} (50% maximal effective dose; ED_{50}) and was 0.1 μM for EMP1 in both cell lines. The amount of EPO that induced 50% maximal proliferation was established to be ca. 10 pM for the native human receptor line and ca. 1 pM for the truncated human receptor line. The ability of each experimental peptide to induce cell proliferation was determined in a series of similar experiments on the two different cell lines as indicated in the text and shown here are some examples of typical proliferation curves. One can easily see the improved proliferative potential of EMP16 and the differential effects of EMP8 which is much more active on the truncated receptor bearing line. The extracellular ligand binding domain is the same for both cell lines and the K_d of EPO identical at ca. 0.27 nM and the number of receptors nearly identical at 850 and 1050 for the full-length and truncated receptor lines, respectively.

tion of 4.4% DMSO and 0.007% TFA. These samples were incubated for 4 h at room temperature and stored at 4 °C for 12 h before analysis on reducing and nonreducing SDS−PAGE.

RESULTS

Peptide Competitive Binding and EPO-Responsive Cell Proliferation. To understand the structural features of EMP1 that are critical for binding and activation of the EPO receptor, we employed two different binding assay formats. These assays measure the ability of the parent peptide or variants to compete for [^{125}I]EPO binding to immobilized EBP or to cell-associated EPO receptors. Respectively, these represent the extracellular ligand binding domain of the EPO receptor immobilized on agarose beads or membrane bound receptors. Functionally, only the latter format should allow free receptor dimerization.

EMP1 competes for binding to immobilized EBP with an IC_{50} of 5 μM ± 2 (Figure 2). We have previously reported that the apparent K_d for the EPO−EBP interaction is ca. 5 nM (*10*). The binding affinity is consistent with the EPO−EBP affinity value for other EBP preparations produced in CHO cells or bacteria of 1−2 nM (*12, 13*). The EMP1 value is about 25-fold higher than the 0.2 μM reported initially (*6*). However, the antibody immobilized extracellular domain of the EPOR used in ref *6* displayed an EPO receptor binding affinity of 200 pM, suggesting that a systematic difference in the assay formats gives rise to the binding difference which is 25-fold for both EPO and EMP1 (beads vs antibody immobilization). This difference may well reside in the potential of the two systems to allow receptor dimerization which may freely occur in the case of antibody immobilization but would not be likely with the receptor immobilized on beads. This idea is supported by studies with cell-associated EPO-receptors on TF-1 cells where the EMP1 IC_{50} was determined to be 0.07 μM (Figure 2), a value 2.5-fold lower than that reported (*6*). These data are consistent with the idea that EBP and TF-1 cell derived values are, respectively, representative of monomer and dimer binding modes.

The ability of the peptide variants to support cellular proliferation as an indicator of receptor activation was studied in two different EPO-responsive cell lines that express alternative forms of the human EPO receptor. The first expresses the native form of the human receptor and the second a truncated form that has been manipulated to delete the 40 amino acid C-terminal residues of the receptor shown to contain negative regulatory control elements (*14, 15*). This truncation reduces the intracellular region from 225 to 185 residues. It was expected that the resultant cell line would be hypersensitive to EPO and, by analogy, to EPO mimetics due to the loss of the negative regulatory control region. The truncated receptor cell line was indeed found to be about 10 times more sensitive to EPO with an ED_{50} value of 1 pM versus 10 pM for the full-length receptor (data not shown). However, the truncated receptor line was not more sensitive to EMP1 and which displayed identical potential on the two cell lines (Figure 3). In contrast, it is more sensitive to some of the EMP1-derived peptides reported below.

To compare data obtained with the two cell lines, results from peptide proliferation studies were standardized to the number of counts observed at the 50% maximal value

Table 1: EMP1-Based Substitution Series

		binding IC_{50} (μM)		EP0-ED_{50} (μM)	
sequence no.	sequence	EBP beads	TF-1 cells	truncated human receptor	human receptor
EMP1	GGT**Y**SCHF**GP**L**TW**VCK**P**QGG	5	0.07	0.1	0.1
EMP6	GGTASCHF**GP**L**TW**VCK**P**QGG	120	58	IA[a]	IA
EMP7	GGTTSCHF**GP**L**TW**VCK**P**QGG	120	26	IA	IA
EMP8	GGTFSCHF**GP**L**TW**VCK**P**QGG	60	1.5	2.0	>10
EMP9	GGT**Y**SCHFA**P**L**TW**VCK**P**QGG	80	1.0	IA	IA
EMP10	GGT**Y**SCHF**G**AL**TW**VCK**P**QGG	10	4.0	0.1	0.3
EMP11	GGT**Y**SCHF**GP**A**TW**VCK**P**QGG	5	0.08	0.06	0.1
EMP12	GGT**Y**SCHF**GP**LA**W**VCK**P**QGG	90	9	IA	IA
EMP13	GGT**Y**SCHF**GP**LTAVCK**P**QGG	>500	>500	IA	IA
EMP14	GGT**Y**SCHF**GP**LT**F**VCK**P**QGG	30	0.3	0.5	0.1
EMP15	GGT**Y**SCHF**GP**L**TW**VCKAQGG	>100[b]	ND[c]	0.3	10

[a] IA = inactive. [b] Solubility limited. [c] ND = not determined.

obtained with EPO to calculate an EPO ED_{50} (effective dose 50% stimulation) value for individual mimetic peptides. The amount of EPO that induced 50% maximal proliferation varies slightly in interassay variability but was established over the course of many studies (>100) to be ca. 10 pM for the native human receptor line and ca. 1 pM for the truncated human receptor line. A typical EMP1 proliferation study in human receptor bearing cells demonstrates an ED_{50} of 0.1 μM (Figure 3A). Despite the 10-fold lower ED_{50} for EPO in the truncated EPO receptor bearing cells, the ED_{50} for EMP1 in both cell lines was 0.1 μM (Figure 3B), a value to which all derivative peptides were compared. The data were reported as their measured ED_{50} if the peptide displayed adequate proliferative potential to reach the 50% maximum response level of EPO or >10 μM if the peptide retained some proliferative potential but did not reach the 50% level. Peptides which did not induce levels of radioactivity greater than the control without added EPO (a baseline level of intrinsic cellular radioactivity incorporation) were scored as inactive (Figure 3). The EPO ED_{50} is equivalent to an EC_{50} for peptides with full activity curves which include a high concentration plateau such as EMP1, EMP16, etc. For example, the EC_{50} and EPO ED_{50} values for EMP1 are both 0.1 μM. The practical maximal concentration of test peptide in the proliferation assay was 10 μM and was experimentally limited by residual DMSO concentrations or peptide solubility. For these reasons, the data are reported as EPO ED_{50} values since the more traditional value of half-maximal activity of each individual peptide cannot be determined because peptide solubility and residual DMSO concentrations preclude determination of a plateau from which half-maximal activity can be estimated for peptides with reduced activities compared to EMP1.

Alanine Replacement Analysis of EMP1. The original phage selection process and subsequent sequence analysis produced almost 80 distinct sequences that could bind to the ligand binding domain of the EPO receptor (*6*). Several positions within the 20 amino acid sequence were found to appear exclusively or at high frequency as indicated, xxx-YxCxxGPxTWxCxPxxx. These conserved positions were systematically replaced with alanine to determine the effect on both binding and mimetic activity in vitro. These substitutions were made within the background of a single peptide sequence, EMP1. [Note: peptide amino acid sequences are numbered sequentially from the *N*-terminus relative to EMP1 and are designated with a superscript number (i.e., Tyr^4) in the text. Some peptide sequences appear in more than one table for comparison purposes.] Most of the alanine substitutions of conserved residues decreased the competitive binding affinity (Table 1). Trp^{13} (EMP13) had the most significant effect, resulting in almost undetectable binding with a complete loss of mimetic activity. Alanine substitution of Tyr^4, Gly^9, or Thr^{12} each resulted in relative binding losses of manyfold and a concomitant loss of mimetic action. Unexpectedly, substitution of Pro^{10} (EMP10) with alanine had virtually no effect on competitive binding or mimetic activity, suggesting that an acceptable β-turn structure can be achieved in the peptide with an alanine replacement. Substitution of Pro^{17} (EMP15) resulted in greater than 100-fold loss of mimetic activity on native human but only a 3-fold loss on the truncated human receptor, suggesting that the interaction of EMP15 with the extracellular domain of the receptor is different or the proliferative signal induced upon EMP15 binding is processed differently when the intracellular domain is truncated. The relative binding of this peptide was also significantly altered, and measurement was limited by decreased peptide solubility above 100 μM. This proline is not as conserved as other positions in the phage selected sequences (*6*), and the reason for the apparent importance of the residue remains unclear in light of the truncation data described below. Alanine substitution of Leu^{11} had no effect on activity as might be expected for a residue demonstrating significant variability in the phage selected clones.

The two aromatic functionalities at positions Tyr^4 and Trp^{13} appear to play especially important roles in both EPO

Table 2: EMP1-Based Truncation Series

		binding IC_{50} (μM)		EP0-ED_{50} (μM)	
sequence no.	sequence	EBP beads	TF-1 cells	truncated human receptor	human receptor
EMP1	GGT**Y**SCHF**GP**L**TW**VCK**P**QGG	5	0.07	0.1	0.1
EMP16	GGT**Y**SCHF**GP**L**TW**VCK**P**Q	8	0.07	0.02	0.0115
EMP17	**TY**SCHF**GP**L**TW**VCK**P**QGG	40	0.07	0.8	0.3
EMP18	**TY**SCHF**GP**L**TW**VCK**P**Q	30	2.5	7	1
EMP19	**Y**SCHF**GP**L**TW**VCK**P**	45	9	>10	>10
EMP20	**Y**SCHF**GP**L**TW**VCK	70	8	4	8
EMP21	**Y**SCHF**GP**L**TW**VC	>250[a]	30	>10	IA[b]
EMP22	**Y**SCHF**GA**L**TW**VCK	160	ND[c]	IA	IA
EMP23	**Y**-CHF**GP**L**TW**VC	>100	ND	IA	IA
EMP24	SCHF**GP**L**TW**VCK	90	31	IA	IA
EMP25[3]	GGCRI**GP**I**TW**VCGG	65	ND[d]	IA	IA
EMP26	HF**GP**L**TW**V	>500	ND	IA	IA
EMP27	GGT**Y**SC-F**GP**L**TW**VCKPQGG	200	140	IA	IA

[a] Solubility limited. [b] IA = inactive. [c] ND = not determined. [d] Equivalent to AF11154.

receptor binding ability and EPO-mimetic properties of EMP1. To investigate further the significance of these two positions, Tyr[4] was replaced with Phe (EMP8) and Thr (EMP7) while Trp[13] was replaced with Phe (EMP14). The former substitutions were made to investigate the relative importance of the polar functions and aromaticity of Tyr[4]. The Thr substitution was made prior to the elucidation of the crystal structure, which showed the importance of the aromatic ring of Tyr[4]. Threonine substitution (EMP7) resulted in a peptide with properties similar to the alanine substitution with greatly reduced binding and no detectable ability to function as an EPO mimetic (Table 1). In contrast, restoration of aromaticity (EMP8) resulted in the recovery of some relative binding activity and residual mimetic activity on the native human EPO receptor bearing cells while the truncated receptor cells were again somewhat more responsive with an ED_{50} of 2 μM. Restoration of aromaticity at Trp[13] (EMP14) resulted in a significant recovery of activity on both cell lines, to levels similar to EMP1, and demonstrated binding levels of only 6-fold or 4-fold less than EMP1 on EBP or cells, respectively (Table 1). These data indicate that aromatic residues at positions 4 and 13 are important and that the hydroxyl on Tyr[4] has a vital role in EMP1 agonist activity.

From these data, it is clear that while a peptide may have the ability to compete with EPO for receptor binding it does not necessarily result in the ability of the peptide to activate the receptor. Presumably, there are both productive and nonproductive modes of binding. The competitive binding ability of the peptides are significantly different on TF-1 cells (dimer mode) and EBP beads (monomer mode). The widest range for this effect was observed with peptides with altered dimerization properties such as EMP6 (see below) where the binding relative to EMP1 was 24-fold and 820-fold, respectively, for the monomer and dimer modes of binding. The ability of a peptide to promote receptor dimerization appears to be a key feature in the ability to activate the receptor and to compete effectively for cellular EPO receptor binding.

Deletion Analysis of EMP1. We next sought to determine the minimal active structure within the EMP1 sequence by truncating the *N*-proximal and *C*-terminal portions of the peptide outside the disulfide bonded loop structure (Table 2). Deletion of the two C-terminal glycines (EMP16; Figure 3) resulted in a sequence with improved proliferative properties, while deletion of only the *N*-terminal glycines (EMP17) had a net negative effect on both binding and bioactivity. Deletion of the four *N*- and *C*-terminal glycines (EMP18) had a net negative impact on both binding and bioactivity relative to EMP1. Further truncation down to a sequence with two residues on either side of the cysteines (EMP19), demonstrated very poor activity with 10 μM peptide retaining less than 50% of EPO activity on both cell lines while binding was only impacted by about 9-fold for monomer binding and 128-fold for TF-1 binding. This loss of receptor activation activity could be partially restored by deletion of the C-terminal proline of EMP19 to obtain EMP20 (Figure 3). This peptide sequence represents the minimal structure with >50% activity that we have identified to date. Deletion of the *C*-terminal lysine (EMP21) resulted in a peptide whose activity measurements were limited by poor solubility properties in these experimental conditions but with some residual activity on the truncated line, suggesting that an active epitope exists within the less soluble peptide. Within the context of the EMP20 sequence, which retained binding and proliferative potential, deletion of the *N*-terminal tyrosine (EMP24) resulted in a peptide with no detectable potential to support proliferation of EPO respon-

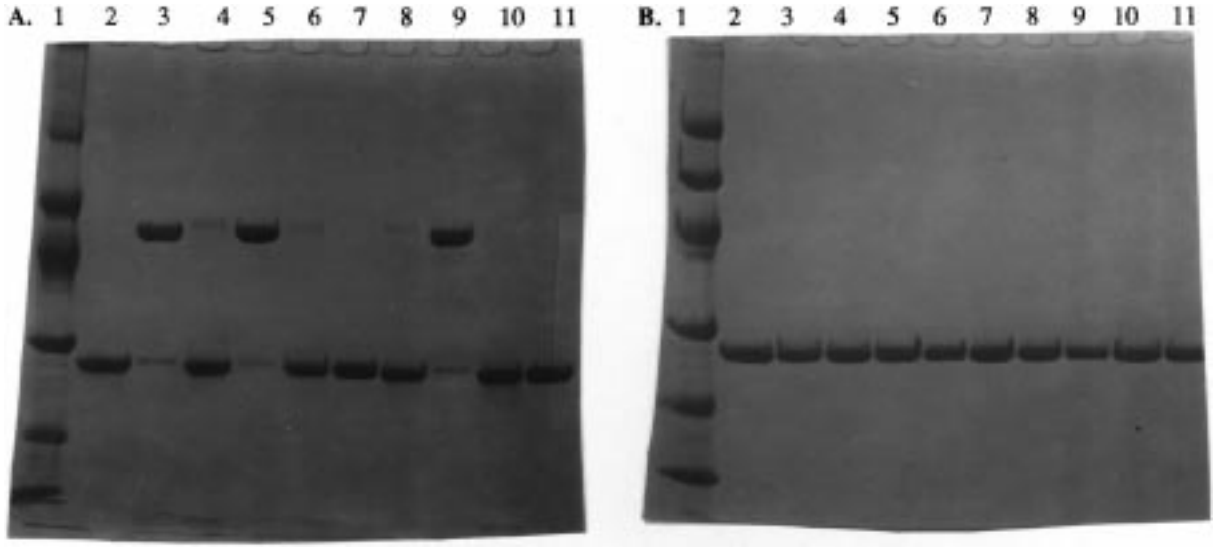

FIGURE 4: DPDPB stabilization of EBP dimerization by truncated EPO mimetic peptides. EBP (22 μM) was incubated in the presence or absence of DPDPB (1.1 mM) and 400 μM of peptides EMP1, EMP20, EMP24, and EMP16 in 75 μL of PBS, pH 7.5, with all reactions and controls containing identical reaction buffers. The samples were incubated for 4 h at room temperature and stored at 4 °C for 12 h before analysis (5 μL of each sample) on nonreducing (panel A) and reducing SDS−PAGE (panel B). Lane 2, EBP, 5 μg; lanes 3 and 4, EMP1 with and without DPDPB; lanes 5 and 6, EMP20 with and without DPDPB; lanes 7 and 8, EMP24 with and without DPDPB; lanes 9 and 10, EMP16 with and without DPDPB; lane 11; EBP and DPDPB, no peptide. Lane 1 contains molecular mass markers of 14.4, 20.1, 30, 43, 63, and 94 kDa in both nonreduced and reduced SDS−PAGE experiments and the sample order is the same. EMP 20 and EMP24 differ by the absence of Tyr^4 in EMP24 which results in no ability to support the formation of dimer product. Note that a trace of dimer forms in the absence of cross-linker in each case when peptide is present but that no dimer product is produced in the absence of peptide (lane 11, panel A). The reducing gel (panel B) demonstrates that all dimers formed were freely reversed by reduction.

sive cells (Figure 3) and TF-1 competitive binding ability 442-fold less than EMP1 (Table 2) but retained monomer binding properties (18-fold reduction relative to EMP1). The EBP binding potential of EMP24 corresponded well with the binding activity of the initial 14 amino acid receptor binding sequence derived from phage display (*6*), here designated EMP25.

To probe the functional difference between EMP20 and EMP24 we carried out an analysis of the ability of the these peptides to dimerize EBP in solution using DPDPB cross-linking analysis (*7*) and found that the two peptides differ dramatically in their ability to stabilize the EBP dimer (Figure 4). While EMP1 and EMP20 produced considerable receptor dimer, EMP24 produced little observable cross-linked product. The EBP IC_{50} competitive binding values of the two (EMP20 vs EMP24, Table 2) differ by only 4-fold on EBP, yet the ability to dimerize EBP is completely different. The most dramatic difference (442-fold less than EMP1) is the inability of the EMP24 sequence to compete for EPO binding in the TF-1 cell-based binding format. We also examined the ability of the more active EMP16 (Figure 4) to promote receptor dimerization and qualitatively see little difference from EMP1. It is interesting to note that the truncated EMP20 peptide provides an EBP dimerization signal similar to EMP1 and EMP16, indicating that the dimerization study is relatively insensitive to peptide affinity under these experimental conditions.

The above results suggested that the most critical determinant for EPO-mimetic activity outside the disulfide-bonded turn structure appeared to be the Tyr at position four of the EMP1 parent sequence and that this appears to be related to receptor dimerization. To investigate if some positional flexibility exists with regard to Tyr^4 and the central peptide core, EMP23 was synthesized. This 11 amino acid peptide sequence which included only the core structure and an *N*-proximal tyrosine residue was found to be inactive. Given that the tyrosine residue is effectively displaced relative to the central core, this finding is not particularly surprising, but the loss of binding was unexpected. We have not yet investigated whether placement of the free amino-terminal charge has an effect, although EMP24 would suggest not, at least in binding ability on EBP.

The substitution of Pro^{10} with alanine within the context of the EMP1 sequence (EMP10) had virtually no effect on activity (Table 1). To investigate the role of this residue in the minimal active sequence, EMP22 was synthesized with a proline to alanine substitution within the context of the active EMP20 sequence (Table 2). This peptide was found to be unable to support cell proliferation and bound 30-fold more poorly than EMP1 or EMP10 on EBP. It does not dimerize EBP in solution using DPDPB (data not shown). Thus, the peptide structure may be stabilized in a different fashion or adopt an unacceptable conformation in the shorter sequence compared to the longer EMP1 sequence or fall below an affinity sufficient for receptor activation. Structural studies will be required to address this question.

Our initial studies showed that the EMP1 class of peptides must be cyclized to possess mimetic activity (*6*). We sought to confirm that the sequence between the disulfide had no intrinsic binding activity by preparing sequence EMP26. This short linear sequence appears to have no activity in either binding or cell proliferation. In an initial inquiry into the sensitivity of the structure to deletion between the cysteines, EMP27 was synthesized. Within the context of EMP1, His^7 was deleted to obtain a peptide which was inactive in cell proliferation studies yet surprisingly had some residual binding activity on EBP, with an IC_{50} about 40-fold higher than EMP1 and 2000-fold greater than EMP1 on TF-1 cells

Table 3: Position 4 Substitution Series

sequence no.	(X)	sequence	binding IC_{50} (μM) EBP beads	binding IC_{50} (μM) TF-1 cells	EP0-ED_{50} (μM) truncated human receptor	EP0-ED_{50} (μM) human receptor
EMP1		GGT**Y**SCHF**GP**L**TW**VCK**P**QGG	5	0.07	0.1	0.1
EMP6		GGTASCHF**GP**L**TW**VCK**P**QGG	120	58	IA[a]	IA
EMP7		GGTTSCHF**GP**L**TW**VCK**P**QGG	120	26	IA	>10
EMP8		GGTFSCHF**GP**L**TW**VCK**P**QGG	60	1.5	2.0	>10
EMP28	D-Tyr	GGT**X**SCHF**GP**L**TW**VCK**P**QGG	>500	13	>10	>10
EMP29	*p*-NO_2-Phe	GGT**X**SCHF**GP**L**TW**VCK**P**QGG	85	ND[b]	0.8	>10
EMP30	*p*-NH_2-Phe	GGT**X**SCHF**GP**L**TW**VCK**P**QGG	90	2.5	3.0	IA
EMP31	*p*-F-Phe	GGT**X**SCHF**GP**L**TW**VCK**P**QGG	40	0.08	0.1	1.0
EMP32	*p*-I-Phe	GGT**X**SCHF**GP**L**TW**VCK**P**QGG	70	ND	0.3	>10
EMP33	3,5-dibromo-tyr	GGT**X**SCHF**GP**L**TW**VCK**P**QGG	50	15	IA	IA

[a] IA = inactive. [b] ND = not determined.

(Table 2). The crystal structure of EMP1/EBP shows that the next residue, Phe[8], is involved in a hydrophobic core assembly in the complex consisting of both receptor and peptide residues. This deletion may shift the hydrophobic register and likely accounts for the reduced binding and inability to activate receptor.

Replacement of Tyr[4] with Unnatural Amino Acids. To investigate more fully the role of Tyr[4], a series of peptides incorporating unnatural amino acids at this position were constructed (Table 3). The D-Tyr substitution (EMP28) resulted in a sequence almost completely inactive in monomer binding activity (EBP), greatly impacted TF-1 binding ability (185-fold), and demonstrated limited ability to activate either the native or truncated human EPO receptor. The structural difference between EMP1 and EMP28 is the stereochemical configuration (D vs L) of a single amino acid with the effect on both activity and binding being significant with only a trace of proliferative activity remaining. It is likely that this change would cause a significant local structural perturbation. The 3,5-dibromo-Tyr substitution peptide (EMP33) was not active on any of the cell lines but retained some binding activity with an IC_{50} only 10-fold greater than EMP1 but with a TF-1 cell binding value of 214-fold less than EMP1. Again, this indicates that the presentation of the ligand binding domain can have rather extreme effects on the ability of a given peptide to compete for EPO binding.

The Tyr[4] hydroxyl appears to serve as a hydrogen bond acceptor for the amide backbone NH of receptor residue Ser[92] (*7*). A series of four substitutions was then made to probe the role of the hydroxyl group in the receptor−peptide interaction, by replacing the *p*-hydroxyl position with NO_2, NH_2, F, and I (EMP29-EMP32, respectively; Table 3). The amine substitution demonstrated only weak proliferative activity on truncated receptor, while the p-F-Phe derivative retained modest levels of both binding and receptor activation potential. These observations are consistent with the ability of the substituents at the *para* position to serve as hydrogen bond acceptors since the amine should not be able to perform this function.

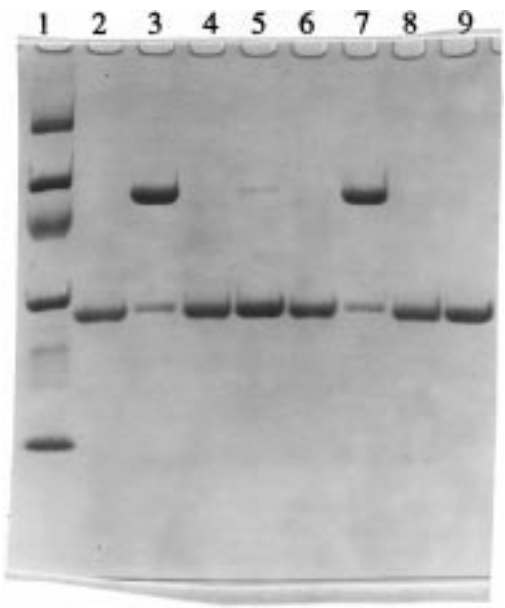

FIGURE 5: DPDPB stabilization of EBP dimerization by Tyr[4] substituted EPO mimetic peptides. EBP was incubated in the presence or absence of DPDPB and peptides EMP1, EMP6, and EMP32 as described in the Figure 4 legend and examined on nonreducing SDS−PAGE. Lane 2, EBP, 5 μg; lanes 3 and 4, EMP1 with and without DPDPB; lanes 5 and 6, EMP6 with and without DPDPB; lanes 7 and 8, EMP32 with and without DPDPB; lane 9; EBP and DPDPB, no peptide. Lane 1 contains molecular mass markers as in Figure 4. EMP32 has the ability to induce EBP dimerization and has mimetic activity while EMP6 does not have the ability to activate receptor nor stabilize EBP dimer formation. The peptides EMP32 and EMP6 contain *p*-I-Phe and Ala substitutions at the Tyr[4] position, respectively.

The ability of these Tyr[4] variants to dimerize EBP in solution was investigated by comparing the ability of the position 4 Ala and the *p*-I-Phe-substituted peptides to form receptor dimer by DPDPB cross-linking (Figure 5). These data illustrate that the inactive alanine-substituted peptide (EMP6) has little ability to support EBP dimerization while the significantly less active (EMP32) appears to possess dimerization ability similar to that of EMP1. Thus, the

Table 4: Sequence Variants and Analogs of EMP1

		binding IC_{50} (μM)		EP0-ED_{50} (μM)	
sequence no.	sequence	EBP beads	TF-1 cells	truncated human receptor	human receptor
EMP1	GGT**Y**SCHF**GP**L**TW**VCK**P**QGG	5	0.07	0.1	0.1
EMP34	Ac-GGT**Y**SCHF**GP**L**TW**VCK**P**QGG	20	0.03	0.06	0.01
EMP35	GGL**Y**ACHM**GP**M**TW**VCQ**P**LGG	3	0.1	0.08	0.1
EMP36	GGL**Y**ACHM**GP**M**TW**VCQ**P**LRG	1	ND[a]	ND	0.1
EMP37	LGRK**Y**SCHF**GP**L**TW**VCQ**P**AKKD	5	0.1	0.15	0.1
EMP38	TIAQ**Y**ICYM**GP**E**TW**ECR**P**SPKA	5	ND	0.02	0.1
EMP39	GGT**Y**SEHF**GP**L**TW**VKK**P**QGG[b]	>100	ND	IA[c]	IA

[a] ND = not determined. [b] Cyclic via an amide bond between side chains E6 and K15. [c] IA = inactive.

qualitative ability of a Tyr^4 peptide variant to support dimerization as monitored by DPDPB cross-linking corresponds to the ability to induce proliferation.

Evaluation of Sequence Analogues of EMP1. To explore additional structure−function relationships and search for sequences with enhanced binding or proliferative potential, several other peptides were synthesized (Table 4). One simple modification was acetylation of the *N*-terminus (EMP34; Table 4) that led to a sequence with improved proliferative potential of up to 10-fold on the native receptor line but without significantly altered EBP binding properties. Alternative phage display systems were employed to discover sequences with improved binding properties (N. Wrighton, unpublished results), and these peptides were used to determine if the sequence variants had increased proliferative capacity. Two of the sequences (EMP35 and EMP36; Table 4) appeared to possess marginally improved affinity (EBP) but did not result in improved proliferative potential since their respective ED_{50} values are similar to that of EMP1. The activity of two other more highly charged peptide sequence variants, EMP37 and EMP38, differ little from EMP1 suggesting that substitutions outside the core and Tyr^4 have little effect on peptide activity and structure (Table 4). It is important to note that EMP35, EMP36, and EMP38 all have Met at the Phe^8 equivalent position demonstrating that other residues at this site are functionally well tolerated.

Finally, in an attempt to define the nature of the structural importance of the disulfide bond (Figure 1), a variant of EMP1 was constructed which was cyclized via an amide bond between the side chains of a glutamic acid residue at position 6 and a lysine at position 15. This sequence, EMP39 (Table 4), was essentially inactive in both binding and cell proliferation studies. The disulfide bond appears to play a significant role in the hydrophobic assembly which occurs between the two peptides in the crystal structure. It is unclear if the polar amide bond replacement is responsible for the lack of activity of this peptide or the substantial increase in size of the cyclic linker from $R-CH_2-S-S-CH_2-R$ to $R-CH_2-CH_2-CO-NH-CH_2-CH_2-CH_2-CH_2-R$ (Figure 1) since either may disrupt the hydrophobic register of the peptide dimer assembly. Additional investigation will be required to evaluate more carefully the effect of the size of the cyclic portion of the mimetic peptide relative to binding and activation of the receptor.

DISCUSSION

Our dissection of the EMP1 primary sequence has focused on the functional role of residues which were found to be strictly or highly conserved in the original family of phage display selected sequences. Of the strictly or highly conserved residues within the EMP1 structure, xxxYxCxxGPxTWxCxPxxx, only Pro^{10} could be substituted with alanine without a significant impact on binding or bioactivity. These observations suggest that the phage display process primarily selected amino acids that were more critical for receptor binding than residues required for or limited by phage presentation constraints. The phage surface presentation of the selected peptides appears not to have been limited by any intrinsic limitations induced by a fusion protein. It still remains unclear exactly how the phage display system selected peptides which are receptor agonists.

Gly^9 and Pro^{10} within EMP1 occupy the i and $i + 1$ positions of a slightly distorted type I β-turn with proline in a trans orientation as shown in Figure 6 (7). In the $i + 1$ position of β-turns, proline is highly preferred, although other residues such as alanine can be accommodated (*16*). On the other hand, at the i position, residues such as asparagine and aspartate are highly preferred in type I β-turns with glycine being present in this position but less frequently (*16*).

The Pro to Ala substitution is well tolerated in the 20-mer (EMP1) but not in the truncated peptide (EMP22). The proline side chain itself does not make significant contact (Figure 6) with the EPO receptor which is also indicated by EMP10 binding. Hence, the difference in binding and activity of EMP22 must be attributed to some inability to stabilize the peptide conformation when in a truncated form. Other truncated peptides of equivalent length (EMP20) show a marked decrease in binding and activity, even with glycine and proline at position i and $i + 1$. Hence, the further decrease in activity by alanine substitution at $i + 1$ in the truncated peptide is not easily interpretable until the conformational preferences of these shorter peptides are known including the ability to dimerize, as well as an analysis of the end effects through introduction of a positive charge closer to the important binding residues in the core.

Other conserved residues include Gly^9 and Thr^{12}, which also participate in the β-turn at positions i and $i + 3$, respectively (Figure 6). Substitution of either of these

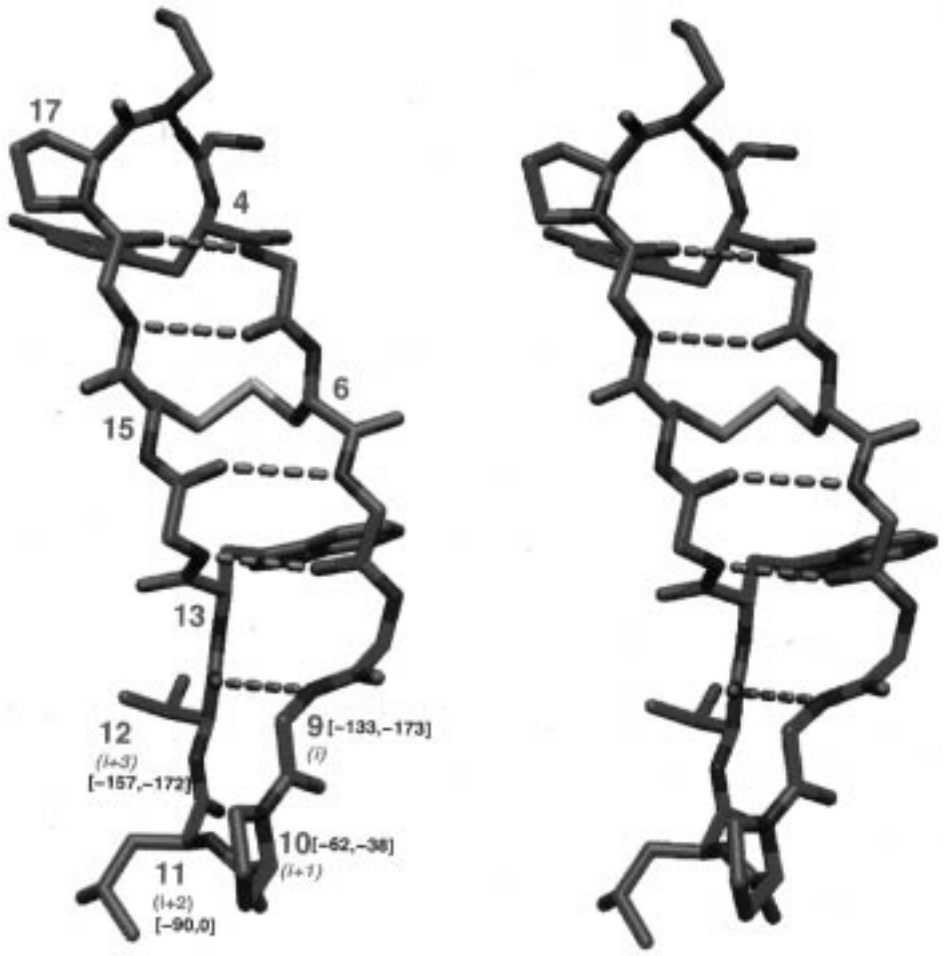

FIGURE 6: The monomeric structure of EMP1 molecule as observed in the EBP-EMP1 complex. For clarity, only the side chains of the disulfide bridge, Tyr^4, Phe^8, Gly^9, Pro^{10}, Leu^{11}, Thr^{12}, and Trp^{13} are displayed. The positions and the ϕ, φ values of the β-turn residues (Gly^9, Pro^{10}, Leu^{11}, Thr^{12}) are marked in magenta and blue, respectively. The number of mainchain/side chain contacts of these four residues with the receptor molecule are 5/0, 7/0, 8/2, and 1/5 for residues 9, 10, 11, and 12, respectively. The intramolecular main-chain hydrogen bonds are colored in orange and show their role, in stabilizing the peptide conformation in addition to the disulfide bridge.

residues with alanine (EMP9 and EMP12) results in a complete lack of proliferation activity and in a substantial decrease in competitive binding. A main-chain−main-chain hydrogen bond is formed in the β-turn between the Gly^9 amide nitrogen and Thr^{12} carbonyl oxygen. In addition, the threonine side-chain hydroxyl (Oγ) hydrogen bonds back to its own main chain at residue Trp^{13}, which may help stabilize the conformation of the peptide. Otherwise, neither residue has significant interaction with the receptor, so abolition of activity by substitution of these residues would appear to be a result of perturbation of peptide conformation itself that might propagate sufficiently to alter interactions of important contact residues with the receptor. Although a glycine is not a required residue at the *i* position in type I β-turns, substitution by alanine might reduce its main-chain flexibility and its ability to form hydrogen bonds that stabilize the β-turn and β-sheet conformation of the peptide.

Alanine substitution of Tyr^4 and Trp^{13} have drastic effects on competitive binding (decrease of 24- and >100-fold, respectively, on EBP; 828- and >7000-fold, respectively, for TF-1 cells) and result in peptides devoid of mimetic activity. Incorporation of a smaller polar residue at position 4 has a similar effect (EMP7), but restoration of aromaticity (EMP8) results in partial recovery of activity. Reestablishment of aromaticity at position 13 (EMP14) leads to almost complete recovery of both binding and mimetic activity. Both Tyr^4 and Trp^{13} are involved in a hydrophobic core that appears critical for peptide dimerization and receptor binding as illustrated in Figure 1 (*7*). In the 2:2 EBP−EMP1 complex, Tyr^4 is the only peptide residue that is involved in a side-chain specific hydrogen bond with the receptor. These observations reinforce the critical role that hydrophobic packing interactions play in the association of these mimetic peptides with the EPO receptor (*7*).

Within the series of truncated peptides, several interesting observations can be made. The first concerns the flanking glycines which were present in the peptide not through selection via the phage display process but rather by their use as linkers in the original phage display fusion construct (*6*). These glycines are disordered in the EMP1-EBP crystal structure and, therefore, might not be expected to play a significant role in the activity of the peptide (*7*). However, deletion of the *C*-terminal glycine pair (EMP16) results in a modest increase in activity on both the truncated and native human EPO receptor bearing lines of 5- and 8.5-fold, respectively. Conversely, truncation of the *N*-terminal glycine pair (EMP17) had a net negative effect on activation of both cell lines as well as on receptor binding. Furthermore, the truncation of both sets of glycines (EMP18) had a relatively substantial effect on proliferative activity with ED_{50}

Table 5: Peptide Intramolecular and Peptide−Peptide Intermolecular Hydrogen Bonds

intramolecular[a] peptide1−peptide1	intermolecular[b] peptide1−peptide2
Thr^{3}O-Gln^{18}N	Tyr^{4}O-Cys^{6}N
Ser^{5}N-Lys^{16}O	Tyr^{4}N-Cys^{6}O
Ser^{5}N-Lys^{16}N	Cys^{6}O-Tyr^{4}N
His^{7}N-Val^{14}O	Cys^{6}N-Tyr^{4}O
His^{7}O-Val^{14}N	
Gly^{9}N-Thr^{12}O	

[a] Main-chain hydrogen bonds found in the β-hairpin structure of one of peptide monomers in the dimer. Equivalent interactions are seen in peptide 2. [b] Main-chain hydrogen bonds found in the four-stranded β-sheet that constitutes the dimer polar interface of the two monomeric peptides as seen in the EMP-EMP1 complex (adapted from ref *7*).

values, decreasing by 70- and 10-fold on the truncated and native human EPO receptor lines, respectively. Obviously, these residues, which were originally placed in the phage linker to enhance flexibility and presentation, play a role in the overall activity of the peptide with effects on both binding and mimetic activity. The structural role, if any, played by these residues remains unclear.

Further *N*-terminal truncations up to Tyr^4 resulted in peptides with decreasing ability to compete for EPO binding and decreased ability to function as EPO mimetics. The deletion of peptide residues outside the cyclic peptide core would eliminate a number of hydrogen bonds that are found in both peptide−peptide and peptide−receptor interactions (Figure 6 and Table 5). Tyr^4 must be retained in any truncated EMP1 derivative in order to function as an EPO mimetic, as illustrated by comparison of the binding and mimetic potential of EMP20 with that of EMP24. EMP20 functions as a minimal effective agonist structure capable of the induction of cell proliferation with relative binding 14-fold less than EMP1 on EBP and 114-fold less on cells. EMP24, which differs from EMP20 only by loss of the Tyr^4 equivalent, was observed to be inactive at concentrations of up to 10 μM, but demonstrated binding at a level of 18-fold less than EMP1 and only 4-fold less than EMP20 on EBP. The binding effect of EMP24 on TF-1 cells was much more dramatic with EMP24 binding falling by 442-fold compared to EMP1 and 4-fold less than EMP20. These data indicate that within the two binding modes it is much more difficult for an EMP1 variant to compete in a system which allows receptor dimerization. Thus, Tyr^4, can almost be regarded as an on−off switch for mimetic activity when attached to the conserved cyclic peptide core. Functionally, this appears to be related to the ability of the peptide to dimerize the receptor as detected with DPDPB and compete effectively in the TF-1 binding assay.

Perhaps, even more significant is the observation that EMP20 can serve as an EPO receptor agonist and at 13 amino acids represents a considerably smaller structure than the 20 residue sequence of EMP1. Thus, even within this novel 20 amino acid mimetic sequence a smaller agonist epitope can be identified. These data further illustrate that only a few critical interactions are required for ligand binding events (ref *17* and references therein) and, in this case, for effective receptor activation. As has been suggested previously (*18*−*20*), these minimal functional epitopes may contain sufficient information for their subsequent reduction to small molecule agonist compounds. The 13 amino acid agonist peptide EMP20 at a molecular mass of about 1500 Da is within reach of small molecule design and can serve as a minimal model for an EPO receptor agonist. One significant hurdle will be the design of a small molecule capable of the dimerization of the receptor, a step that appears to be critical for receptor activation. The EMP1-EBP crystal structure demonstrated an almost perfect 2-fold symmetrical arrangement of the receptor and mimetic peptide which might reduce the difficulty of this task since the design process can be viewed as a "one site" problem with similar binding sites on each erythropoietin receptor. However, the crystal structure of growth hormone (GH) complexed with the extracellular domain of its cognate receptor (*21*) revealed a nonsymmetrical arrangement whereby one growth hormone molecule interacts with two receptors using different sites on the ligand in an asymmetric fashion. Asymmetry is also likely to be present when EPO is complexed with its receptor, since the EPO structure itself is unlikely to be symmetrical. Therefore, the ligand-based design of EPO or GH small molecule mimetics would be expected to be somewhat simplified by using a smaller symmetrical structure such as EMP1. Recently, peptide agonists of the thrombopoietin receptor (*22*) have been identified, suggesting that it may be possible to obtain peptide ligands for other hematopoietic receptor superfamily members such as growth hormone.

The importance of Tyr^4 to the activity of EMP1 was demonstrated in both the alanine replacement and truncation series and was further expanded by incorporation of non-natural amino acids at this critical position (Table 3). The information gained with this series of substitutions was somewhat difficult to understand completely, but some generalizations are possible. The stereochemical configuration of this residue was important for both binding and proliferative activities, while loss of the hydroxyl, or the aromatic side chain, were also critical. Nitro-, fluro-, and iodo- substructures are able to substitute, to a certain extent, for the hydroxyl with the smallest, fluorine, having activity which approached that of EMP1; all are capable of serving as H-bond acceptors. More extensive modification such as that represented by the 3,5-dibromo-Tyr resulted in an inactive peptide. DPDPB cross-linking studies further indicated that Tyr^4 also is a critical element in receptor dimerization. Receptor dimerization detected with DPDPB appears to correspond to receptor activation ability. It is possible that other peptide-mediated receptor dimerization modes, not detectable with DPDPB, could lead to receptor dimerization or that receptor dimerization without activation may also occur.

The crystal structure shows that Tyr^4 is found in a central hydrophobic core, which consists of several residues including the key receptor amino acid Phe^{93} (*7*). Previous alanine mutagenesis studies have demonstrated the importance of this residue in the binding and activation of the receptor by EPO (*23*, *24*). This observation is consistent with the notion that EMP1 and EPO may share overlapping binding areas and similar receptor activation strategies via dimerization. Further, we have shown that the other hydrophobic residues at the Phe^{93} site restores some EPO binding potential to EBP, consistent with the suggestion that hydrophobic interactions, mediated in part by Phe^{93}, are critical for ligand binding (*24*). A large hydrophobic side chain is present at this equivalent receptor position in both the GH and EPO receptors (*18*,

24, 25), suggesting that this is a critical region to which the design of potential therapeutic modifiers might be targeted.

We also examined the ability of various peptides to support the proliferation of two different cell lines which expressed different forms of the human EPO receptor. EMP1 was found to stimulate both native and truncated cell lines with an equivalent ED_{50}. The truncated human receptor line appears to be hypersensitive to EPO, as expected, but not EMP1. This observation was extended to many of the EMP1 variants, in that substitutions or deletions that destroyed or limited activity were not significantly better stimulators of the hypersensitive cell line. However, two peptides (EMP28 and EMP30, Figure 3 and Table 3) were inactive on the full-length receptor-containing cell line but retained sufficient activity to determine an ED_{50} on the hypersensitive line. Significant differences in response between the full-length human and truncated human receptor cell lines were also observed with EMP8 and EMP15 (Figure 3 and Table 1) in which a phenylalanine and an alanine substitution were made for Tyr^4 and Pro^{17}, respectively. These substitutions decreased the ED_{50} by about 100-fold on the full-length human-receptor-bearing cell line, yet only decreased the truncated line ED_{50} by 20-fold and 3-fold for EMP8 and EMP15, respectively. Additional nonnatural substitutions of Tyr^4 exemplified by EMP28-32 (Table 3, Figure 3), continue this differential trend where the hypersensitive truncated receptor bearing line is more tolerant of substitutions. On the other hand, EMP33 in which Tyr^4 is replaced with 3,5-dibromotyrosine is completely inactive on both cell lines despite displaying significant binding activity on EBP and TF-1 cells. The EPO equilibrium binding constant for both cell lines has been determined (data not shown) and the K_d. value for EPO is identical at ca. 0.27 nM and the number of receptors nearly identical at 850 and 1050 for the full-length and truncated receptor lines, respectively. Taken together, these data suggest that subtle differences exist in the mechanism of receptor activation even though the two human receptors share exactly the same ligand binding domain. The origin of the activation difference is unknown and may arise due simply to the lack of a functional negative regulatory region on the human truncated receptor. Further, competitive binding analysis with EPO does not appear predictive of the ability of a given peptide to activate the receptor. However, other mechanisms might also explain this difference in receptor sensitivity including an active conformation of slightly different architecture in the truncated and full-length human EPO receptors or in differing potential of the two receptors to achieve an active conformation for effective signal transduction to occur. It is important to note that the bulk of the peptides which exhibit this behavior are Tyr^4 variants, the critical residue related to receptor dimerization potential.

In summary, we have previously shown that the function of the 34 kDa EPO hormone can be mimicked by a much smaller peptide of ca. 2 kDa and that the sequence of the mimetic is unrelated to EPO (*6*). The binding site of the mimetic peptide likely overlaps those receptor regions that are also employed for EPO binding (*7*). Here, we report that conserved residues found within one member of the family of EPO-mimetic peptides have important roles in both binding, receptor dimerization and in mimetic activity. Further, the finding that a 13 amino acid variant of the original 20 amino acid sequence can function as an EPO receptor agonist suggests that it may be possible to further reduce this sequence into an even smaller minimal agonist epitope. Structural knowledge of this 13 amino acid sequence might be expected to simplify the design of a small molecule therapeutic.

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