Symmetric Signaling by an Asymmetric
1 Erythropoietin : 2 Erythropoietin Receptor Complex

by

Yingxin Zhang

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Submitted to the Department of Biological Engineering in partial fulfillment of the requirements for the degree of

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SYMMETRIC SIGNALING BY AN ASYMMETRIC 1 ERYTHROPOIETIN : 2 ERYTHROPOIETIN RECEPTOR COMPLEX

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Submitted to the Department of Biological Engineering on May 23, 2008, in Partial Fulfillment of the Requirements for the Degree of Master of Engineering in Biomedical Engineering

ABSTRACT

One erythropoietin molecule binds asymmetrically to two identical receptor monomers via erythropoietin site 1 and site 2, although it is unclear how asymmetry affects receptor activation and signaling. Here we report the computational design and experimental validation of two mutant erythropoietin receptors: one that binds only to erythropoietin site 1 but not site 2, and one that binds only to site 2 but not site 1. Expression of either mutant receptor alone in Ba/F3 cells cannot elicit a signal in response to erythropoietin, but when co-expressed, there is a proliferative response and activation of the JAK2 Stat5 signaling pathway. A truncated erythropoietin receptor with only one cytosolic tyrosine (Y343), on only one receptor monomer is sufficient for signaling in response to erythropoietin, regardless of the monomer on which it is located. The same results apply to having only one conserved juxtamembrane hydrophobic L253 or W258 residue, essential for JAK2 activation, in the full-length receptor dimer. We conclude that despite asymmetry in the ligand-receptor dimer interaction, both sides are competent for signaling, and we suggest that the receptors signal equally.

Thesis Supervisor: Harvey F. Lodish
Title: Professor of Biology and Biological Engineering
Acknowledgments

I would like to thank my parents and my brother for their support throughout my entire time at MIT. I would not have had the opportunity for this amazing education without their unconditional love and encouragement. My parents have made great sacrifices for me to have the life I do, and it is to them that I dedicate this work. They have been my constant support and my guiding light. They have given me invaluable advice and shared with me perspectives that only a rich life experience can shape.

My experiences as both an undergraduate and graduate student here would also have been much less colorful without the wonderful classmates and professors that I have met over the years. They have been peers, mentors, and inspirations to me. When I first came to MIT for undergraduate school, I realized that my class (the Class of 2007) would be the last class to graduate from MIT without the option for a biological engineering major. The Master of Engineering in Biomedical Engineering program (MEBE) has given me the opportunity to pursue research and coursework related to bioengineering, and through it I have experienced a great deal of personal growth, as the transformation from an undergraduate to a graduate student has been a memorable journey. I am very grateful to the BE staff for guiding me through this rewarding program.

MIT has always been a place where research at all levels is encouraged from the moment we step onto the campus. The late Vernon Ingram was the first to take me under his wings at MIT, and helped nurture my interests in science and research, despite my lack of experience. His passion for science and teaching really touched me, and I miss him dearly. Another former research mentor I would like to give my sincere thanks to is Geoffrey von Maltzahn, whose excitement is contagious. Geoff was not only a teacher to me, but also a treasured friend. His bright mind, his patience, and his resourcefulness are all reasons why he is my role model.

The work presented here is the culmination of an idea that was born from the collaboration between the Lodish Lab and Bruce Tidor’s lab, bridging computational modeling with protein engineering to overcome obstacles in studying a complex system in nature. The computational work described herein was performed by former chemistry PhD student Mala Radhakrishnan in the Tidor Lab as part of her thesis. I am grateful to both Mala and Bruce for their contributions to this project; the computational work stands as the foundation to this piece of research. I would also like to acknowledge former Lodish lab members: Xiaohui Lu for his teaching and guidance in the early stages of this project, and Alec Gross for discussions and help with the binding affinity assays. To the other members of the Lodish Lab, thank you all for your help along the way, as colleagues, friends, and mentors. I could not have hoped for a better group of labmates to have spent my past two years with. And to the Whitehead Institute, for supporting graduate students in Whitehead labs and making this experience possible for me.

Finally, my greatest thanks goes to my supervisor, Harvey Lodish. Always a teacher at heart, he took me into his lab generously as his first ever Masters student and patiently worked with me in developing this project. He challenges me to think, to question, and to achieve – all things that have helped me grow in my time here and have given me the confidence to succeed in what I previously never thought was possible. I am honored to have worked with such a brilliant man, a teacher who has always been there to guide me, but who also encouraged me to explore the possibilities.
Introduction

Erythropoietin (Epo) is a cytokine necessary for regulating erythropoiesis (Graber et al. 1978). Produced primarily by the kidney in adult humans (Jacobson et al. 1957), it stimulates erythroid progenitor cells to proliferate and terminally differentiate by interacting with cell surface erythropoietin receptors (EpoRs) to initiate downstream signaling cascades (Koury et al. 1988). Although EpoR is mainly expressed in hematopoietic tissues, it is also expressed in vascular endothelial cells (Anagnostou et al. 1994) and tissues of the central nervous system (Liu et al. 1997).

EpoR is a member of the type 1 superfamily of single-transmembrane cytokine receptors, all of which share conserved fibronectin III-like extracellular subdomains, a WSXWS motif important for protein folding, and short stretches of conserved cytoplasmic regions termed Box1 and Box2, while lacking intrinsic protein tyrosine kinase activity (Bazan 1990). EpoR in particular associates with JAK2, a member of the Janus kinase family (Witthuhn et al. 1993), following EpoR synthesis in the endoplasmic reticulum. The EpoR is expressed on the cell surface as a pre-formed receptor homodimer (Livnah et al. 1999), mediated by the leucine zipper of the transmembrane domain (Constantinescu, Keren et al. 2001). Binding of Epo to EpoR induces JAK2 activation via either auto- or trans-phosphorylation of the appended JAK2 molecules, and requires the presence of three conserved hydrophobic “switch” residues in the EpoR juxtamembrane domain, L253, I257, and W258 (Constantinescu, Huang et al. 2001). JAK2 activation is necessary for subsequent phosphorylation of up to eight tyrosine residues on the cytoplasmic segment of each EpoR monomer (Klingmuller 1997). These phosphotyrosines recruit downstream signaling molecules for intracellular pathways.
involving Stat5, Ras/MAPK, and PI3K/Akt1 (Miura et al. 1994; Damen, Cutler et al. 1995; Damen, Wakao et al. 1995).

As a monomeric, asymmetric molecule, Epo employs two different interfaces, termed site 1 and site 2, to bind to the two monomers of an EpoR homodimer. Site 1 on Epo is a high affinity binding site, with a $K_D$ of 1nM, whereas site 2 has a thousand-fold lower affinity for the EpoR, with a $K_D$ of ~1µM (Philo et al. 1996). Because the EpoR dimer is comprised of two identical transmembrane proteins, both monomers have the ability to bind Epo at site 1 and site 2, depending on the order and direction in which the Epo molecule enters the receptor binding site. A crystal structure of one Epo molecule bound to two EpoR extracellular ligand binding domains shows that the site 1 interface, which is characterized by a central hydrophobic binding pocket, contains many more overall Epo-EpoR contacts than the site 2 interface, whose interacting residues mostly fall within a subset of the site 1 residues (Syed et al. 1998).

It is hypothesized that the asymmetry of the Epo-EpoR interaction is necessary for proper orientation of the EpoR monomers to initiate JAK2 activation (Constantinescu, Huang et al. 2001; Seubert et al. 2003). In this scheme, Epo activates EpoR by positioning the two EpoR monomers such that the weakly active catalytic site of one associated JAK2 molecule is adjacent to the critical tyrosine residue in the activation loop of the second JAK2, thus promoting phosphorylation of the tyrosine and activation of the second JAK2 to initiate downstream signaling events (Lu et al. 2006). Epo mimetic peptides activate EpoR by forming symmetric EpoR dimers, although the level of activation and response achieved by these peptides is inferior to Epo stimulation (Livnah et al. 1996; Wrighton et al. 1996). This difference suggests that there is an optimal
receptor orientation achieved by the Epo binding interaction. Furthermore, certain peptides that bind and dimerize EpoR do not induce any receptor activation, consistent with the notion that receptor dimerization alone is insufficient for receptor activation and signaling (Livnah et al. 1998). There is also a possibility that the asymmetric interaction between Epo and the EpoR monomers could translate to differences in each monomer’s contribution to the activation of and signaling by the receptor. In particular, the large difference in the Epo binding affinities of site 1 and site 2 may be indicative of a dominant role in the receptor that binds Epo at site 1, or there may be a time delay between the activation of each receptor. However, it is also possible that despite the asymmetry of ligand-receptor interaction, the two monomers contribute equally to the activation and signaling of the EpoR.

Our goal is to systematically explore the mechanism of EpoR activation as a result of asymmetric interaction with Epo, and to understand the role of contributing structural elements in each receptor in initiating JAK2 activation and subsequent signaling. Currently, experimental study of the roles of each individual EpoR monomer in the Epo-EpoR complex is impossible because the monomers have the same molecular structure and cannot be selectively tracked or manipulated. Similar problems in the past have been approached by the construction of “single-chain” dimers, in which a polypeptide linker connects the C-terminus of one monomer to the N-terminus of another such that intramolecular dimerization interactions are strongly favored (Robinson et al. 1996). To implement such a strategy here would require that the linker pass through the membrane and still would be unlikely to cause Epo to bind in a unique orientation, and thus we adopted a different approach. Here, we have computationally designed a pair of
EpoRs mutant in their ability to bind Epo at only site 1 or site 2. Expressed alone, these mutants cannot support proper activation and signaling in response to Epo, but when co-expressed, the response to Epo is restored to nearly wild-type levels. Using this novel cell-based, heterodimeric EpoR system, we generated EpoR mutants to study the contribution of several structural components when present on either the site 1 or site 2-binding monomer. A study of EpoR with such fine control has not been possible with previously existing methods, and we believe this study demonstrates the usefulness of these novel probes as tools for gaining a deeper understanding of other homodimeric cytokine receptors such as the G-CSF and thrombopoietin receptors.
Results

Computational Design of EpoR Mutants

Our goal was to generate a pair of human EpoRs each able to bind only to either site 1 or site 2 on Epo, mutants that would allow us to differentiate between the two receptors in an Epo-binding receptor complex. To design these mutants, we computationally analyzed mutations at candidate residues in the binding sites of EpoR to predict changes in Epo binding properties.

From the 1.9-Å crystal structure of Epo complexed with the extracellular portion of wild-type EpoR (PDB ID: 1EER) (Syed et al. 1998), we identified candidate residues whose mutation could predominantly affect only site 1 or site 2, but not both. Figure 1a shows the structure of two EpoR monomers bound to one Epo molecule, with the site 1 and site 2 EpoR interaction regions highlighted in yellow.

Of EpoR residues interacting directly with Epo at site 1 but not site 2, His114 and Glu117 were chosen for initial mutational analysis. His114 was chosen because previous work (Radhakrishnan 2007) showed that mutating this residue can significantly alter binding affinity; the binding affinity of EpoR H114K and EpoR H114T to Epo were reduced several-fold compared to wild-type EpoR. Glu117 was chosen because it forms a salt bridge with Epo residue Arg150, a critical site 1 interaction residue. A mutation at this site could disrupt electrostatic complementarity and thereby reduce binding affinity. In the site 2-binding EpoR monomer, His114 and Glu117 are both too far from Epo residues for direct packing or hydrogen-bonding interactions, although they are potentially close enough for intermediate-range electrostatic interactions. Thus
computational mutational analyses of the two residues were performed at both site 1 and site 2 to assess these potential effects.

The site 2 interface contains fewer EpoR residues than site 1, with those at site 2 mostly a subset of those at site 1. We selected EpoR Met150 for our initial mutational analysis because it makes close contacts at the site 2 interface but not at site 1. Thus, we hypothesized that an appropriate mutation could disrupt binding to Epo site 2 with less effect at site 1.

The three selected EpoR residues, His114, Glu117, and Met150, were each computationally mutated in turn at either the site 1 or site 2 interfaces and the effect on binding affinity evaluated, with the goal of identifying mutations that would specifically alter binding free energy at one site while leaving the other site relatively unaffected. For each candidate residue at its primary binding site (site 1 for His114 and Glu117, and site 2 for Met150), mutations to all other amino acids were considered except to cysteine, proline, and glycine, in order to avoid disulfide bridge interference and changes in backbone flexibility and conformation. Those amino acids that were predicted to greatly weaken binding at the primary binding site were then evaluated for binding to the other Epo site.
Figure 1. Structures of Epo complexed with wild-type and mutant EpoR dimers. (A) One monomer of wild-type EpoR binds to Epo at site 1 while a second wild-type monomer binds at site 2. The highlighted yellow regions on EpoR distinguish the site 1 and site 2 interaction residues, which are not mutually exclusive. In the wild-type EpoR homodimer, both monomers are capable of binding Epo site 1 and site 2. (B) Epo is bound to a functional EpoR heterodimer formed from a pair of mutant EpoRs each specific for binding at only Epo site 1 or site 2. The site 1-null mutant EpoR (S1·EpoR) containing mutations H114K and E117K binds only to Epo at site 2, while the site 2-null mutant EpoR (S2·EpoR) containing mutation M150E binds only to Epo at site 1. When S1· and S2· EpoRs form a heterodimer on the cell surface, they should bind Epo in a specific manner as shown above and behave as a functional receptor capable of activation and signaling. The emphasized side chains on each monomer are important for binding to Epo at each site, and are mutated (not shown) on the opposite monomer. (C) Location of EpoR residues His114 and Glu117 in site 1 and site 2 of the Epo-EpoR complex. His114 is shown in red, Glu117 is shown in blue. Both residues interact directly with Epo at the site 1 interface but are at the periphery of the site 2 interface. Mutation of Hist114 has been previously shown to significantly weaken binding by several-fold. Glu117 forms a salt bridge with Epo residue Arg150 at site 1. (D) Location of EpoR residue M150 in site 1 and site 2 of the Epo-EpoR complex. M150, shown in green, forms intricate hydrophobic packing interactions with Epo residues at site 2, but not at site 1. M150 is present within the interfacial regions of both sites, but it is much more buried upon binding at site 2.

Computational Analysis of Candidate Site 1 EpoR Mutants
Figure 1c shows the locations of His114 and Glu117 in the asymmetric 1-ligand:2-receptor complex; they interact directly with Epo at the site 1 interface but are at the periphery of the site 2 interface. Table 1 shows the computed change in the Epo-EpoR binding free energy, relative to wild-type EpoR, as a result of mutating either His114 or Glu117 to other amino acids at site 1 and leaving site 2 unaltered. For both residues, mutation to either of the basic amino acids Lys or Arg yields the highest computed disruption in binding affinity, over 3.0 kcal/mol in both cases. The primary mechanism of disruption is via unfavorable electrostatic interactions, as shown in Table 1. Interestingly, mutation of Glu117 to any other amino acid considered was predicted to be unfavorable, a result suggesting that an acidic residue at position 117 is important for binding Epo. Structural analysis supports this conclusion, as Glu117 forms a salt bridge with Epo Arg150.

Because the Epo-EpoR interaction at site 1 is high-affinity, more than one mutation might be necessary to ensure defective binding. Table 2 shows the predicted change in binding free energy for mutating both His114 and Glu117 to pairs of residues that were individually promising. Because of the relative proximity of the two residues, the changes in binding free energy of the double mutants at site 1 was not the sum of the individual contributions shown in Table 1. For these promising double mutants, we also considered the effect of the mutations on receptor binding at site 2. As Table 2 shows, mutating both His114 and Glu117 to basic residues was predicted to abrogate site 1 binding without greatly affecting site 2 binding. In this analysis we considered a binding free energy change of greater than +3.0 kcal/mol to be significant, based on previous experimentally-tested designs (Radhakrishnan 2007). Of these four predictions, we chose
to experimentally synthesize the H114K / E117K double EpoR mutant. In comparing the repacked crystal structure of wild-type EpoR site 1 to the double mutant, both His114 and Glu117 interact with Arg150 on Epo. Mutation of these groups to lysines is predicted to be highly destabilizing electrostatically because it puts multiple positively charged groups across the interface from one another. Thus we expect that the EpoR H114K / E117K mutant will disrupt site 1 binding and allow only for binding to site 2 on Epo.

<table>
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<td>Y</td>
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*Because van der Waals interactions are very sensitive to small movements in side chains, we hypothesized that the extremely high binding free energy changes for mutations to Phe, Tyr and Trp could be relieved by backbone and side-chain relaxations not modeled within the discrete search. Indeed, these high energy changes disappeared when computed structures were allowed to minimize after the discrete packing (data not shown).

Table 1. Computed change in Epo-EpoR binding free energy in kcal/mol, relative to wild-type EpoR, for mutants at either EpoR His114 or Glu117 at the site 1 interface, or Met150 at the site 2 interface. For site 1 mutations, site 2 was left intact during calculations, and vice versa. The letters in the left-most column represent the amino acid of the resulting mutation at either position 114, 117, or 150 on EpoR. Along with the total change in
binding free energy, the electrostatic (Elec) component and the van der Waals component (vdW) of the total free energy change are also shown. Positive values of free energy change are indicative of unfavorable interactions relative to wild-type EpoR, while negative values represent favorable interactions.

**Computational Analysis of Site 2 Mutant EpoR Candidates**

Figure 1d shows the locations of Met150 in the two EpoR monomers of the asymmetric complex. At the site 2 interfaces, Met150 interacts with multiple Epo residues in a hydrophobic region. At site 1, Met150 does not directly make close contacts with Epo but is still within 5.0 Å of multiple Epo residues. Table 1 shows the predicted change in Epo-EpoR binding free energy, relative to wild-type EpoR, upon mutation of Met150 at the site 2, leaving site 1 unmutated. In Table 2, site 1 calculations are shown for those mutations predicted to disrupt site 2 binding. The mutations fall into two classes. The first class included mutation to bulky, aromatic residues, Phe, Trp, and Tyr, which cause disruption by unfavorable van der Waals interactions. However, these unfavorable interactions might be relieved with modest relaxation and thus were not ideal candidates. The second class of disruptive mutations altered the charge of the side chain: Asp, Glu, Lys, and Arg. Because electrostatic effects are less amendable to relaxation, these predictions were considered more robust, and selections were made from among them for experimental testing.

We chose the EpoR M150E site 2 mutant for experimental synthesis and analysis, as it was predicted to significantly disrupt binding at site 2 with no significant effect at site 1 (Table 2). The predicted mutant structures at the site 1 and site 2 interfaces show that at site 1, mutation to Glu allows for potential long-range electrostatic interactions with Arg162 on Epo, and loses only minor packing interactions with Epo residues. At site
2, however, the mutation to Glu is disruptive. In our calculations the negative charge remains buried, although other conformational possibilities are also unfavorable.

<table>
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*The extremely high binding free energy changes for mutations to Phe, Tyr and Trp disappeared when computed structures were allowed to minimize after the discrete packing.

Table 2. Comparison of site 1 and site 2 computed change in Epo-EpoR binding free energy in kcal/mol, relative to wild-type EpoR, for candidate site 1-null and site 2-null EpoR mutants. For site 1 mutations, site 2 was left intact during calculations, and vice versa. Along with the total change in binding free energy, the electrostatic (Elec) component and the van der Waals component (vdW) of the total energy change are also shown. Positive values of free energy change are indicative of unfavorable interactions relative to wild-type EpoR, while negative values represent favorable interactions. An ideal candidate mutant has a large positive value at the primary site of interaction (site 1 for H114 and E117, site 2 for M150) and a value close to zero at the other site.

Validation of Mutant EpoR Candidates in Ba/F3 Cells

Based on our computational predictions we selected the human EpoR double mutant H114K / E117K (which we will hence forth call S1- EpoR) predicted to be deficient in site 1 binding and the single mutant M150E (S2- EpoR) predicted to be deficient in site 2 binding for experimental validation. We hypothesized that if both
mutant receptors were deficient in binding as predicted, then expression of either mutant in cell culture would not elicit a response to Epo stimulation, whereas co-expression of both receptors could lead to a reconstitution of response to Epo, based on the formation of functional receptor heterodimers (Figure 1b). In such a scheme, expression of either mutant alone results in the formation of homodimers on the cell surface of monomers either both deficient in site 1 binding (S1'/S1') or in site 2 binding (S2'/S2'), and thus unable to form a 2 receptor complex with Epo. On the other hand, when the mutants are co-expressed, formation of heterodimers (S1'/S2') would lead to pairs with a site 1 deficient monomer that still has an intact site 2, and a site 2 deficient monomer that has an intact site 1, thus providing the means for the heterodimeric EpoR to bind to Epo in a specific manner.

Figure 2a shows the Epo-dependent proliferation of Ba/F3 cells expressing either wild-type EpoR, S1' EpoR, S2' EpoR, or co-expressing both S1' EpoR and S2' EpoRs. As expected, cells expressing either the S1' EpoR or S2' EpoRs did not proliferate in response to any Epo concentration tested, similar to control Ba/F3 cells not expressing any EpoR. Importantly, and as we anticipated, cells co-expressing EpoR S1' and EpoR S2' exhibited an Epo growth response comparable to cells expressing wild-type EpoR.

Western blotting shows similar levels of wild type, S1' EpoR, and S2' EpoRs (Figure 2c). FACS analysis of surface expression of EpoR using an antibody specific for the HA epitope tag appended to the extracellular N-terminus of EpoR (Figure 2b) shows comparable surface expression levels of wild-type, S1', and S2' EpoRs. Thus lack of proliferation of Ba/F3 cells expressing only EpoR S1' or S2' mutant EpoRs is not due to reduced cell surface expression of the mutant receptors.
Figure 2. Co-expression of S1<sup>−</sup> and S2<sup>−</sup> EpoR mutants in Ba/F3 cells restores concentration-dependent proliferation in response to Epo. (A) Assay for proliferative response of EpoRs to Epo stimulation. Ba/F3 cells expressing various EpoRs were cultured at 50,000 cells/ml in RPMI media containing 10% FBS and varying concentrations of Epo (0.01, 0.1, 1, and 10 units/ml). Relative viable cell density was measured using an MTT assay in
duplicate after 72 hours of growth. Data are represented as mean +/- standard deviation. (B) Surface expression of wild-type and mutant EpoRs. FACS analysis was performed on Ba/F3 cells expressing wild-type or mutant HA-tagged EpoR in an IRES-GFP containing vector. Whole cells were stained with mouse α-HA monoclonal primary antibody followed by APC-tagged goat α-mouse secondary antibody to probe for surface receptors. GFP versus APC signal is plotted, with medians as shown. (C) Overall expression of EpoRs as assayed by Western blotting. Ba/F3 cells expressing various EpoRs were grown in IL-3-containing media, then harvested and lysed for SDS-PAGE. EpoR was detected with rabbit polyclonal α-EpoR antibody.

**Epo Binding Affinity of Mutant Epo Receptors**

To test the effect of the computationally selected mutations in EpoR on Epo binding, we measured equilibrium binding of $^{125}$I-Epo to the surface of Ba/F3 cells expressing wild-type, S1’, or S2’ EpoR (Figure 3). Analysis of Epo binding to Ba/F3-wild-type EpoR cells indicated there were ~2,100 surface Epo binding sites per cell [$B_{max} = 3.54$ fmoles/$10^6$ cells (95% conf. interval: 3.47, 3.61)], with $K_D = 0.14$ nM (95% conf. interval: 0.13, 0.15), which is similar to previous measurements of the affinity of Epo for cell surface EpoR. Although Ba/F3-S1’ EpoR and Ba/F3-S2’ EpoR cells expressed similar amounts of HA-tagged EpoR protein on the cell surface compared to Ba/F3-wild-type EpoR cells (Figure 2b), Epo bound with a lower affinity to cells expressing the mutant receptors (Figure 3). Ba/F3-S1’ cells specifically bound only a very small amount of Epo compared to wild-type EpoR cells; binding was so low that the $K_D$ for the S1’ EpoR could not be reliably determined. Ba/F3-S2’ cells bound Epo with a reduced, but measurable, $K_D = 0.9$ nM (95% conf. interval: 0.6, 1.2). Given that Epo site 1 has a much higher binding affinity for EpoR than Epo site 2 (Philo et al. 1996), the above results suggest that, as predicted, the mutations in the S1’ EpoR disrupt high affinity Epo site 1 binding, and the mutations in the S2’ EpoR disrupt low affinity Epo site 2 binding.
Figure 3. Epo-binding by wild-type or mutant EpoRs. Equilibrium binding of $^{125}$I-Epo to the surface of parental Ba/F3 cells (no EpoR) or Ba/F3 cells expressing wild-type or $S_1$- or $S_2$-mutant EpoR was measured. Binding to parental Ba/F3 cells (no EpoR) represents non-specific binding. Bound and free $^{125}$I-Epo was measured and binding curves fitted through the plotted data points as described in Experimental Procedures. Estimated dissociation constants are as follows: wild-type EpoR ~ 0.14 nM, $S_1$-EpoR ~ 77 nM, and $S_2$-EpoR ~ 0.90 nM.

Hydrophobic Switch Residues on Either Receptor Monomer Contribute Equally to EpoR Activation

Three conserved hydrophobic residues in the juxtamembrane cytosolic domain of EpoR, L253, I257, and W258, are necessary for the activation of the associated JAK2.
Mutating any of these residues to alanine in the EpoR homodimer dramatically suppresses growth and signaling responses to Epo stimulation but does not affect the ability of the Epo receptors to bind Jak2, traffic normally to the cell surface, or bind Epo (Constantinescu, Huang et al. 2001). Here we ask whether these switch residues have a greater contribution to EpoR activation when they are present on the site 1 or site 2-binding EpoR monomer. In the experiments described in Figure 4, we studied both S1- and S2- EpoRs that had either the L253A or W258A mutation.

Figure 4a shows that having the inactivating L253A mutation on either monomer, but the normal L253 on the other, does not abrogate the proliferative response to Epo, but rather reduces the growth response at Epo concentrations between 0.01–1.0 units/ml; this is true whether the L253A mutation is present on the S1-binding EpoR: S1- EpoR expressed together with S2- EpoR L253A, or on the S2-binding receptor: S1- EpoR L253A expressed together with S2- EpoR. The maximal growth response is similar to that of wild-type EpoR at a saturating concentration of 10 units/ml. Surface expression of the L253A EpoR mutants is approximately 1/3 of wild-type EpoR (data not shown), and therefore likely accounts for some of the reduced response to Epo stimulation. As expected cells expressing mutant EpoRs in which the L253A mutation is present in both monomers, S1- EpoR L253A and S2- EpoR L253A, do not proliferate in the presence of Epo.
Figure 4. Conserved hydrophobic residues are needed only on one monomer of EpoR to achieve a proliferative response to Epo. (A) L253A mutation on either EpoR monomer causes symmetric partial impairment of response to Epo stimulation. Ba/F3 cells expressing various EpoRs were cultured at 50,000 cells/ml in RPMI media containing 10% FBS.
and varying concentrations of Epo (0.01, 0.1, 1, and 10 units/ml). Relative viable cell density was measured with MTT assay performed in duplicate after 72 hours of growth. Data are represented as mean +/- standard deviation. (B) W258A mutation on either EpoR monomer causes symmetric partial impairment of response to Epo stimulation. Ba/F3 cells expressing various EpoRs were cultured at 50,000 cells/ml in RPMI media containing 10% FBS and varying concentrations of Epo (0.01, 0.1, 1, and 10 units/ml). Relative viable cell density was measured with MTT assay performed in duplicate after 72 hours of growth. Data are represented as mean +/- standard deviation.

Similar results were obtained by mutating another conserved hydrophobic residue, W258, to alanine. Figure 4b shows that having the inactivating mutation W258A on either monomer but the normal W258 residue on the other allows proliferation in response to Epo, but ~10-fold higher Epo concentrations are required compared to cells expressing S1' and S2' EpoRs both with a tryptophan at position 258. This is true whether the W258A mutation is present on the S1-binding EpoR: S1' EpoR expressed together with S2' EpoR W258A or whether it is present on the other monomer, S1' EpoR W258A expressed together with S2' EpoR. As expected, cells expressing mutant EpoRs in which the W258A mutation is present in both monomers, S1' EpoR W258A and S2' EpoR W258A, do not proliferate in the presence of Epo. Surface expression of S1' EpoR W258A and S2' EpoR W258A mutants was about half that of wild-type EpoR (data not shown). That mutating W258 to alanine has a greater effect than mutating L253 was expected; mutation of W258 to alanine in otherwise wild-type EpoR inhibits JAK2 phosphorylation completely, whereas EpoR L253A exhibits very low levels of JAK2 activation compared to wild-type EpoR (Constantinescu, Huang et al. 2001).

Taken together, these results show that the critical L253 and W258 residues need be present on only one EpoR in the heterodimeric 1 Epo: 2 EpoR complex in order to activate signaling and proliferation in Ba/F3 cells. The contribution of these residues is the same regardless of which monomer they reside on.
A single tyrosine, Y343, on either the site 1-defective or the site 2-defective EpoR is sufficient to support Epo-dependent cell proliferation and Stat5 activation.

Of the eight conserved tyrosines on the cytoplasmic tail of EpoR that become phosphorylated following JAK2 activation, phospho-Y343 is specific for binding the Stat5 SH2 domain, resulting in phosphorylation of Stat5 by JAK2 (Damen, Wakao et al. 1995). Activated Stat5 then dimerizes in the cytoplasm and translocates into the nucleus to act as a transcription factor for various genes suppressing apoptosis and promoting proliferation and differentiation (Socolovsky et al. 1999). A truncated EpoR containing only amino acids 1–375 (EpoR-H), and retaining only the first phosphorylated tyrosine of the cytoplasmic tail (Y343), supports Epo-triggered proliferation when expressed in IL3-dependent cells (Miura et al. 1991). Mutation of Y343 to phenylalanine in EpoR-H, called EpoR-H Y343F, abolished the ability of EpoR-H to activate Stat5 and significantly depressed the ability of the EpoR to support cell proliferation, indicating the importance of phosphoY343 and Stat5 activation in EpoR signaling and cell survival (Li et al. 2003). To test whether a single phosphorylated tyrosine, Y343, was sufficient if present on either the site 1-defective or the site 2-defective EpoR, we first constructed and tested two truncated EpoRs, one defective in site 1 binding and the other defective in binding Epo site 2, called S1⁻ EpoR-H and S2⁻ EpoR-H. Figure 5a shows that cells expressing both truncated EpoRs together, S1⁻ EpoR-H and S2⁻ EpoR-H, supported Epo-dependent cell proliferation similar to cells expressing two full-length EpoRs, S1⁻ EpoR and S2⁻ EpoR. Cell surface expression analysis by FACS indicated a nearly two-fold higher surface expression of the truncated EpoRs compared to full-length receptors (data not
Figure 5. Truncated EpoR with one Tyr343 is sufficient for proliferation in response to Epo stimulation. All H and H Y343F mutants are truncated EpoRs containing only the first 375 amino acids from the N terminus. (A) Full-length wild-type EpoR is compared to full-length heterodimeric EpoR (S1 / S2), truncated wild-type EpoR 1-375 (EpoR-H), and truncated
heterodimeric EpoR 1-375 (S1·H/S2·H) in a proliferation assay to assess truncated EpoR response to Epo. Ba/F3 cells expressing various EpoRs were cultured at 50,000 cells/ml in RPMI media containing 10% FBS and varying concentrations of Epo (0.01, 0.1, 1, and 10 units/ml). Relative viable cell density measured with MTT assay performed in duplicate after 72 hours of growth. Data are represented as mean +/- standard deviation. (B) Y343 is only needed on one partner in a truncated EpoR dimer for proliferation in response to Epo stimulation. Ba/F3 cells expressing various EpoRs were cultured at 50,000 cells/ml in RPMI media containing 10% FBS and varying concentrations of Epo (0.01, 0.1, 1, and 10 units/ml). Relative viable cell density measured with MTT assay performed in duplicate after 72 hours of growth. Data are represented as mean +/- standard deviation. (C) Heterodimeric EpoR with only one Y343 residue on either monomer is capable of signaling via Stat5 phosphorylation. Ba/F3 cells expressing the various EpoRs were washed and starved for 4 hours at 37°C in RPMI media containing 1% BSA. They were stimulated with Epo at either 0, 1, or 10 units/ml for 10 minutes at room temperature. Stimulation was stopped with excess cold PBS, and cells were pelleted for lysis and Western blotting. Phospho-Stat5 was detected with rabbit α-pStat5 (pTyr694) antibody. As expected, cells expressing either S1·EpoR-H or S2·EpoR-H alone showed no growth response to Epo (Figure 5b).

The experiments in Figures 5b and 5c show directly that a single tyrosine, Y343, on either the Site 1-defective or the Site 2-defective EpoR, is sufficient to support Epo-dependent cell proliferation and Stat5 activation. Cells co-expressing S1·EpoR-H together with S2·EpoR-H Y343F, in which the single tyrosine Y343 is present only on the site 2-binding EpoR monomer, exhibited a growth response to Epo (Figure 5b) and Epo-dependent activation of Stat5 (Figure 5c) similar to that of cells co-expressing mutant EpoRs in which Y343 is present both on the site 1-binding and the site 2-binding EpoR monomer, S2·EpoR-H and S1·EpoR-H, respectively. Similarly, cells co-expressing S1·EpoR-H Y343F together with S2·EpoR-H, in which Y343 is present only on the site 1-binding EpoR monomer, also exhibited a normal growth response to Epo (Figure 5b) and Epo-dependent activation of Stat5 (Figure 5c).

Thus the location of the single (phospho) tyrosine Y343, whether on the site 1-binding or site 2-binding receptor monomer, does not impact the proliferative response to Epo. Both S1·EpoR-H Y343F expressed together with S2·EpoR-H, and S1·EpoR-H
expressed together with S2' EpoR-H Y343F, have nearly identical response curves to Epo stimulation. As controls, Ba/F3 cells expressing either S1' EpoR-H, S2' EpoR-H, S1' EpoR-H Y343F, or S2' EpoR-H Y343F, or co-expressing the tyrosine-null pair S1' EpoR-H Y343F and S2' EpoR-H Y343F have little to no proliferative response to Epo (Figure 5b) or ability to support Epo-triggered activation of Stat5 (Figure 5c).

Collectively, these results show that the Y343 residue in the EpoR cytoplasmic tail, sufficient for sustaining mitogenic activity in Ba/F3 cells, is only necessary on one monomer of the EpoR dimer to allow signaling through the receptor and activation of Stat5. Thus, our results suggest that both receptors are competent for signaling, and we suggest that the receptors signal equally.
Discussion

The erythropoietin receptor is one of several cytokine receptors that homodimerize and bind to two discrete sites on a monomeric ligand (Frank 2002). Here we have analyzed the amino acid residues on EpoR that bind to these different sites on Epo. Despite a significant overlap in the EpoR residues that make up each binding site, we were able to computationally identify key EpoR residues necessary for specific binding to Epo site 1 and site 2. We generated two mutants: one, termed S1−, that is deficient in binding to Epo site 1 but not site 2, and one, termed S2−, that is deficient in binding to site 2 but not site 1. We showed that each of these mutants has the expected properties when expressed in cultured Ba/F3 cells, through the use of Epo-dependent proliferation assays and determination of KD values via equilibrium binding to Epo. More importantly, we showed that when the S1− EpoR and S2− EpoR are co-expressed, the proliferative response to Epo stimulation is similar to that of wild-type EpoR, indicating functional complementation by the two mutant receptors and signaling by Epo-activated S1−/S2− EpoR heterodimers. Using our EpoR mutants we showed that the conserved juxtamembrane hydrophobic residues L253 and W258, essential for Jak2 activation, as well as the intracellular tyrosine Y343 are only needed on one monomer of an EpoR dimer (regardless of which side) to permit activation and signaling in response to Epo. This study illustrates the potential of using computational protein design to create research reagents with properties designed to facilitate experimental investigation of complex biological systems.
Rational Design

Here we assumed mutations at either site 1 or site 2 produced only local structural change and used computational design to search over mutant sequences and conformations while repacking side chains in the local neighborhood. This is likely to be a good assumption for the well packed interfacial interactions in this ternary protein complex.

The structural data suggested that EpoR M150 primarily interacts with Epo site 2 and not with Epo site 1. EpoR M150 does interact with the aliphatic portion of Epo R10 at the site 2 interface. However, the R10 rotamer chosen for the lowest energy repacked wild type and S2- mutant were identical, so the predicted ΔΔG’s were likely due to localized site 2 effects, and not effects transduced through altered conformations of residue R10.

Previous experiments demonstrated that an EpoR M150A mutant was only slightly less responsive to Epo than wild-type EpoR (Middleton et al. 1999). As shown in Table 1, our initial predictions showed a significant loss of affinity at site 2 with an M150A mutation, although the cost was primarily in the van der Waals component of binding and could be relieved through structural relaxation. Moreover, the predicted binding free energy using the other neutral tautomer of H153 was approximately 1.5 kcal/mol more favorable than the one shown in Table 1. Minimizing structures corresponding to both H153 tautomers yielded structures predicted to bind approximately 3.2-5.5 kcal/mol worse than wild type on average, again with the van der Waals energy being the primary difference. On the other hand, both tautomers of the M150E mutant were predicted to bind to Epo site 2 greater than 6 kcal/mol worse than wild type. With
the binding energy mainly stemming from electrostatic contributions, which are less amenable to relaxation, these predictions are likely to be more robust. Therefore, although our methods may still be overestimating the loss of binding due to an alanine mutation, the predictions are consistent with the idea that an alanine substitution at EpoR M150 can affect site 2 binding, but not as much as the M150E mutant.

**Functional Activation of the EpoR Heterodimer**

We then generated site 1-deficient and site 2-deficient EpoR mutants and showed that they behave as predicted. The results from the proliferation assay and the Stat5 signaling assay both confirm that our mutants are unable to activate and signal when expressed alone. The S1\(^{-}\) EpoR and S2\(^{-}\) EpoR mutants expressed alone have a weak response to Epo stimulation at very high concentrations of 10 U/ml in both the proliferation and Stat5 signaling assays, suggesting that although our mutations strongly disrupt site 1 and site 2 function, they do not completely abrogate binding at high Epo concentrations. However, for the purposes of our study, the lack of response to Epo stimulation at physiologic Epo concentrations of 1 U/ml or less is sufficient for assuming that these receptors are nonfunctional when expressed by themselves.

More importantly, when we co-expressed our mutant S1\(^{-}\) EpoR and S2\(^{-}\) EpoR in the same cells, we saw signaling and proliferation in response to stimulation at physiologic Epo concentrations, validating our computational design of a functional heterodimeric EpoR. These experiments also establish that our EpoR mutations target two distinct aspects of the receptor, in this case binding to Epo site 1 and site 2, such that the two mutant receptors can complement one another to restore a normal receptor
response. Because these mutant receptors are expressed normally on the cell surface, it is unlikely that these mutations interfere with proper folding or intracellular trafficking of the receptors. We assume that when S1\textsuperscript{EpoR} and S2\textsuperscript{EpoR} are co-expressed, both homodimers and heterodimers will form in the absence of Epo, but only heterodimers can be activated by Epo stimulation. The formation of non-signaling homodimers (S1\textsuperscript{EpoR} / S1\textsuperscript{EpoR} and S2\textsuperscript{EpoR} / S2\textsuperscript{EpoR}) in cells co-expressing both mutant receptors condition likely explains why the “EpoR S1\textsuperscript{-} / S2\textsuperscript{-}” Ba/F3 strains exhibit a slightly weaker response to Epo when compared to wild-type EpoR.

**Epo Binding Affinities of Mutant EpoRs**

The binding of a single ligand to two cytokine receptor monomers through the use of two distinct receptor binding sites with different binding affinities was first shown in the human growth hormone (hGH) interaction with its receptor (hGHbp) (Cunningham et al. 1991). The human growth hormone receptor binds to two discrete sites --- site 1 and site 2 on the monomeric hGh ligand. This differs from other receptors such as EGFR that dimerize by binding to a pair of identical homodimerized ligands. The fact that a single binding K\textsubscript{D} is found for both hGH-hGHbp and Epo-EpoR binding suggests that receptor dimerization is occurring on the cell surface. Biophysical measurements showed that this 1-ligand:2-receptor interaction is mediated by two receptor binding sites on Epo --- a high affinity site 1 and a low affinity site 2 (Philo et al. 1996).

Our equilibrium binding data is consistent with Philo et al.’s site 1 K\textsubscript{D} value, because both the wild-type EpoR and the S2\textsuperscript{-} EpoR (the mutant still able to bind to Epo site 1) have a K\textsubscript{D} value for wild-type Epo of \(-1\text{nM.}\) The K\textsubscript{D} for the S2\textsuperscript{-} EpoR is slightly
higher than that of the wild-type EpoR; presumably this is because wild-type EpoR can bind Epo at both site 1 and site 2 whereas the S2\(^{-}\) mutant can bind only to site 1 on Epo. Our S1\(^{-}\) EpoR, which is predicted to be deficient in binding to Epo site 1, shows little or no specific binding to Epo, which is also consistent with our predictions. The equilibrium binding assay is only useful for measuring the site 1 binding affinity, as Epo site 1 has a thousand-fold higher affinity for the EpoR extracellular domain than site 2, whose affinity cannot be meaningfully measured in this manner (Philo et al. 1996). Our S2\(^{-}\) EpoR mutant can be compared to the G120R hGH mutant that blocks site 2 binding to the growth hormone receptor. This GH mutant acts as an antagonist to the hGH-hGHbp complex formation by binding to site 1 only and preventing receptor dimerization (Fuh et al. 1992). The difference is that our M150E mutation is made in the receptor, but this mutation also prevents ligand binding to site 2 and therefore prevents Epo from forming a receptor dimer in a conformation appropriate for receptor activation.

**Hydrophobic Switch Residues and Tyrosine Phosphorylation**

We previously proposed a model where the conserved cytosolic juxtamembrane hydrophobic residues, L253, I257, and W258 facilitate the release of kinase-inhibitory interactions between the JH2 and JH1 domains of the JAK2 molecule appended to the EpoR cytosolic domain (Lu et al. 2008). The JAK2 JH2 pseudokinase domain normally acts as a negative regulatory element that, through physical interaction in the absence of ligand stimulation, keeps the JH1 kinase domain in an inactive state (Saharinen et al. 2000). Mutation of EpoR residues L253, I257, or W258 to alanine leads to loss of JAK2 activation by Epo (Constantinescu, Huang et al. 2001), but these hydrophobic residues
are not needed for the constitutive activation of the JAK2V617F mutant. The V617F mutation lies on one of the two principal interfaces between the JH1 and JH2 domains (Lu et al. 2008), suggesting that the function of L253, I257, and W258 may be to disrupt the interaction between the JH2 and JH1 domains to facilitate JAK2 activation.

However, we do not know whether Jak2 activation is in cis or trans. That is, we do not know whether the conserved L253, I257, and W258 residues activate the Jak2 bound to the same EpoR or the opposite one, or possibly both. Here, we showed that these hydrophobic residues need only to be present on one side of the EpoR dimer to support signaling. In particular we see an Epo-dependent proliferative effect on cells with EpoR possessing L253 or W258 on one monomer only. These results suggest (but do not prove) that only one kinase-active JAK2 is necessary for successful EpoR signaling, albeit at reduced efficiency.

We also showed that only one tyrosine, Y343, is needed on an EpoR dimer to support signaling. Y343 must be phosphorylated by an activated JAK2 on the EpoR before it can recruit downstream signaling molecules, but we do not know whether Jak2 phosphorylates tyrosines on the EpoR cytosolic domain in cis or trans. That is, we do not know whether Jak2 phosphorylates tyrosines on the receptor to which it is bound or the opposite one, or possibly both.

Studies on EGF and FGF receptors indicate that the tyrosine kinases linked to these dimeric receptors are capable of intermolecular transphosphorylation, shown through the expression of kinase-active mutant receptors that are able to phosphorylate tyrosines on kinase-negative mutant receptors (Lammers et al. 1990; Bellot et al. 1991). Another example is found in the EGF receptor family members HER2 and HER3, both of
which are incapable of being activated through ligand stimulation, as HER2 lacks the ability to couple to the downstream signaling cascade while HER3 lacks the kinase activity to phosphorylate cytoplasmic tyrosines. Only when they are co-expressed can HER2 and HER3 heterodimerize and activate downstream signaling pathways, suggesting that transphosphorylation must be taking place in this receptor dimer as well (Holbro et al. 2003). Our EpoR mutants may be useful in studying EpoR auto- or transphosphorylation as they allow us to generate kinase-dead and tyrosine to phenylalanine mutants that may be used to show the cis or trans relationship between the conserved juxtamembrane hydrophobic residues, the appended JAK2 proteins, and the EpoR tyrosines that become phosphorylated.
Experimental Procedures

Preparation of Epo-EpoR Structure

Studies were initiated using the 1.9-Å crystal structure of Epo complexed with the extracellular portion of EpoR (PDB ID 1EER) (Syed et al. 1998). Flipping of asparagine, glutamine, and histidine residues and removal of most crystallographic water molecules was done as explained in (Lippow et al. 2007). Each of the 41 retained water molecules was assigned by inspection to either the ligand binding partner or the receptor binding partner. The HBUILD facility (Brunger et al. 1988) in the CHARMM software package (Brooks et al. 1983) was used to build all hydrogen-atom positions. CHARMM was also used to build the missing side-chain density of Epo residue Arg166 and to terminate the main chain at Asp8 and Thr220 on both EpoR chains with acetamide and N-methylamide groups, respectively, as residues 0–7 and 221–226 of each monomer were not located in the crystal structure experiment. The PARAM22 all-atom parameter set (MacKerell et al. 1998) and the CHARMM-adapted TIP3P water model (Jorgensen et al. 1983) were used. The crystallized proteins in this structure differ in sequence from wild-type proteins at multiple positions (on Epo: N24K, N38K, N83K, P121N, P122S; on EpoR monomers: N52Q, N164Q, A211E), all of which were at least 9.0 Å (and generally much farther) from the closest residue on a binding partner. In addition, the naturally occurring oligosaccharides present on both Epo and EpoR were not modeled, as they were absent from the crystal structure (due to mutated asparagines). In this work, we refer to this model system as wild-type, and we assume that the relative binding free energies of mutation in this system are not significantly different from that of the naturally-occurring wild-type proteins.
Computational Mutation Analysis

The two-stage hierarchical approach of Lippow et al. (2007) was used to calculate binding free energies of mutants. In the first stage, a conformational search over discrete rotamers for mobile and mutated side chains was done to determine stable conformations of the mutated bound states. All side chains (excluding prolines) and crystallographic water molecules containing atoms within 5.0 Å of the native side chain(s) to be mutated were considered mobile in the repacking calculations, and the backbone was considered rigid. A pairwise energy function was used to evaluate structures and the determination of the global minimum energy structure and the next 29 other lowest-energy structures. All energies used in the combinatorial search were computed using the all-atom CHARMM22 force field (MacKerell et al. 1998). Binding was considered rigid; the unbound states were not repacked separately.

In the second stage, the binding and folding free energy of each of the 30 lowest-energy structures for each mutant was re-evaluated using a more accurate energy function that replaced the electrostatics term in the first stage with a continuum electrostatic treatment using the linearized Poisson–Boltzmann equation (LPBE) (Gilson et al. 1987; Gilson et al. 1988; Sharp et al. 1990) with the PARSE parameters (Sitkoff et al. 1994) and added a nonpolar solvation term that was proportional to solvent-accessible surface area (Lippow et al. 2007). One translation of a 257x257x257 grid was used for numerically solving for electrostatic potentials, corresponding to approximately 3 grid units/Å. The predicted binding free energy for each mutant or wild-type structure with the lowest bound-state energy was used to calculate the $\Delta G_{\text{bind}}^0$ values shown here.
Additionally, to assess the robustness of the results for predictions aimed to disrupt binding (H114 and E117 at site 1, and M150 at site 2), conformations for promising mutants and the repacked wild type were energy minimized. Residues that were mobile in the repacking were considered mobile in the minimization, and the corresponding backbone atoms were also minimized. Local minimizations were carried out using the adapted-basis Newton-Raphson method in CHARMM, using a tolerance of \(<1\times10^{-7}\) kcal/mol and a distance-dependent dielectric constant of 4. Binding free energies were then calculated from these minimized structures.

**Cloning of human EpoR variants**

The retroviral vector pMX-IRES-GFP with hemagglutinin epitope (HA)-tagged human EpoR cDNA cloned upstream of the internal ribosome entry site (IRES)-green fluorescent protein (GFP) sequences was a gift from Dr. S.N. Constantinescu (Ludwig Institute for Cancer Research, Brussels, Belgium). The pBI-CD4 vector was kindly provided by Dr. Christopher Hug (Whitehead Institute for Biomedical Research). The site 1 EpoR double mutant (S1' EpoR H114K/E117K) was generated by mutating the CAC codon for histidine 114 of EpoR to AAG for lysine, and the GAA codon for glutamic acid 117 of EpoR to AAA encoding lysine using site-directed mutagenesis as described by the Quickchange strategy (Stratagene). For the site 2 EpoR mutant (S2' EpoR M150E), the AUG codon for methionine 150 of EpoR was mutated to AAG encoding glutamic acid using the same method as in site 1. To generate the L253A and W238A site 1 and site 2 hEpoR mutants, the S1' and S2' pMX-HA-hEpoR-IRES-GFP constructs were used for site-directed mutagenesis via the Quickchange strategy. The CTG codon for
leucine 253 or the TGG codon for tryptophan 258 was changed to GCG (alanine) in each respective mutant.

The truncated hEpoR constructs (EpoR-H) were made using the wild-type pMX-HA-hEpoR-GFP construct described above as template. PCR reactions were performed using primers annealed to the BamHI site before the start of the HA-hEpoR sequence and to the region at hEpoR amino acid 375, with the addition of a stop codon followed by an EcoRI restriction site inserted after the sequence for alanine 375. The purified PCR products were digested with BamHI and EcoRI, then purified again before ligating into the original pMX-HA-hEpoR-IRES-GFP vector digested with BamHI and EcoRI to remove the full-length HA-hEpoR sequence. To obtain the site 1 and site 2 mutant variants (S1\(^{-}\)EpoR H, S2\(^{-}\)EpoR H) of these truncated EpoR constructs, S1\(^{-}\) and S2\(^{-}\) HA-hEpoR inserts were excised from pMX-HA-hEpoR-GFP using BglII restriction sites, and ligated into the truncated wild type EpoR-H pMX-GFP construct also digested with BglII, to replace the wild-type hEpoR sequence with the mutant sequences for S1\(^{-}\) and S2\(^{-}\). To generate EpoR-H constructs containing a Y343F mutation, site-directed mutagenesis via the Quickchange strategy was used on the truncated EpoR-H constructs.

To subclone all the above various EpoR constructs into a pBI-CD4 vector for co-expression, BamHI and NotI restriction sites were used to excise the wild-type and mutant HA-hEpoR sequences from the pMX-IRES-GFP vectors for ligation into the pBI-CD4 backbone.
Expression of EpoR in Tissue Culture Cells

Retroviral vectors carrying wild-type and mutant EpoR cDNAs were transfected into 293T cells along with the pCL-Eco packaging vector, using FuGENE6 transfection reagent (Roche). Supernatant containing retroviruses were collected after 48 hours. Ba/F3 cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine, and 6% WEHI-3B cell conditioned medium as a source of IL-3. To stably express wild-type or mutant EpoRs in Ba/F3 cells, retrovirus supernatants were used for spin-infection of Ba/F3 parental cells. For co-expressing two different EpoR mutants, 50% of each supernatant (one for the pMX-GFP cDNA and one for the pBI-CD4 cDNA) was used to maintain the total viral load. Two days after spin-infection, about 90% of the Ba/F3 cells were GFP positive. For single expressions, all the GFP positive cells were collected with FACS, and for co-expressions, GFP and CD4 double positive cells were collected with FACS.

Surface and Overall EpoR Expression Analysis

To measure surface HA-tagged hEpoR protein, in-tact Ba/F3 cells were stained with mouse anti-HA primary antibody (Covance Research Products, Inc., Berkeley, CA, monoclonal HA.11, clone 16B12) and then with an allophycocyanin (APC)-conjugated goat anti-mouse Ig secondary antibody (BD Biosciences, San Jose, CA). Flow cytometry measurements were made on a FACSCalibur machine, and data were analyzed with CellQuest software (Becton Dickinson, San Jose, CA) to measure the median fluorescence intensities for GFP and APC. Relative overall expression of hEpoR in Ba/F3 cells was determined by Western blotting. Ba/F3 cells expressing various hEpoRs were
grown in RPMI with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine, and 6% WEHI-3B cell conditioned medium as a source of IL-3. They were harvested and lysed with RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for SDS-PAGE. For Western blotting, hEpoR was detected with rabbit polyclonal anti-hEpoR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, EpoR antibody M-20).

**Determination of Binding Affinity**

Recombinant human erythropoietin (Epo) (a gift from Amgen Inc., Thousand Oaks, CA) was labeled with Iodine-125 (Perkin Elmer, NEN, Boston, MA) using Iodo-Gen reagent (Pierce Biotechnology, Rockford, IL). $^{125}$I-Epo specific activity was routinely ~ $4 \times 10^6$ cpm/pmole, representing an average of one $^{125}$I atom incorporated per molecule of Epo. To measure binding affinity, binding medium (fresh Ba/F3 culture medium without any WEHI-3B cell conditioned medium) was equilibrated to atmosphere in a 4°C room and the pH was adjusted to 7.3. Ba/F3 cells expressing various EpoRs were incubated in binding medium with $^{125}$I-Epo for 18 hours at 4°C. Bound and free $^{125}$I-Epo were separated by centrifuging cells through a layer of FBS. Non-specific binding was measured with parental Ba/F3 cells which lack detectable Epo-specific binding sites on their surface (Gross et al. 2006). $K_D$ and $B_{\text{max}}$ were determined by fitting the model for a single-class of non-cooperative binding sites to data obtained over a range of ligand concentrations, using MATLAB software (The Mathworks, Inc., Natick, MA).
**Proliferation and MTT Assays**

IL-3-dependent Ba/F3 parental cells or Ba/F3 cells expressing wild-type or mutant EpoRs were washed and resuspended at 50,000 cells/ml in RPMI with 10% FBS, 1% L-glutamine, and recombinant human Epo (Amgen) at a range of specified concentrations. After three days, 100ul of well-mixed culture from each well was removed in duplicate for a 96-well format MTT assay to determine cell viability and growth, according to established protocols (Promega Corporation, Madison, WI). The signal from the MTT assay was measured as absorbance at 570nm using a 96-well plate reader.

**Phosphorylation Signaling Assays**

Ba/F3 cells expressing various hEpoRs were washed and resuspended at 1x10^6 cells/ml in RPMI with 1% BSA, then starved for 4 hours at 37°C. After starvation, cells were stimulated with a range of specified Epo concentrations in RPMI with 1% BSA for 10 minutes at room temperature. Excess cold phosphate buffered saline (PBS) was added to stop stimulation, followed by pelleting of cells. Pellets were lysed in modified RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1mM Na_3VO_4, 5mM NaF) for SDS-PAGE and Western blotting. Phosphorylated Stat5 was detected with polyclonal rabbit anti-phosphoStat5 (pY694) antibody (Cell Signaling Technology, Inc., Danvers, MA).
References


phosphorylated and activated following stimulation with erythropoietin." Cell 74(2): 227-236.