Short Article

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

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SUMMARY

Via sites 1 and 2, erythropoietin binds asymmetrically to two identical receptor monomers, although it is unclear how asymmetry affects receptor activation and signaling. Here we report the design and validation of two mutant erythropoietin receptors that probe the role of individual members of the receptor dimer by selectively binding either site 1 or site 2 on erythropoietin. Ba/F3 cells expressing either mutant receptor do not respond to erythropoietin, but cells co-expressing both receptors respond to erythropoietin by proliferation and activation of the JAK2-Stat5 pathway. A truncated receptor with only one cytosolic tyrosine (Y343) is sufficient for signaling in response to erythropoietin, regardless of the monomer on which it is located. Similarly, only one receptor in the dimer needs a juxtamembrane hydrophobic L253 or W258 residue, essential for JAK2 activation. We conclude that despite asymmetry in the ligand-receptor interaction, both sides are competent for signaling, and appear to signal equally.

INTRODUCTION

Erythropoietin (Epo) is a cytokine necessary for regulating erythropoiesis. Produced primarily by the kidney in adult humans, it stimulates erythroid progenitor cells to proliferate and terminally differentiate by interacting with cell surface erythropoietin receptors (EpoRs) to initiate downstream signaling cascades (Molineux et al., 2009). EpoR is a member of the type 1 superfamily of single transmembrane cytokine receptors, including the prolactin, G-CSF, thrombopoietin, and growth hormone receptors, all of which share conserved fibronectin III-like extracellular subdomains and a WSXWS motif important for protein folding. All have stretches of conserved cytoplasmic regions termed Box1 and Box2 that associate with members of the Janus kinase family. EpoR in particular associates with JAK2 and is expressed on the cell surface as a preformed receptor homodimer, mediated by the leucine zipper of the transmembrane domain (Constantinescu et al., 2001a).

Binding of Epo to EpoR induces JAK2 activation, which 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 et al., 1995a, 1995b).

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 1 nM, whereas site 2 has a 1000-fold lower affinity for the EpoR with a K_D of ~1 μM (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 direction in which the Epo molecule enters the receptor binding site. 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 et al., 2001b; Seubert et al., 2003).

The asymmetric interaction between Epo and the two EpoR monomers could lead to differences in each monomer's contribution to receptor activation and signaling. 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. In contrast, despite the asymmetry of ligand-receptor interaction, the two monomers could 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
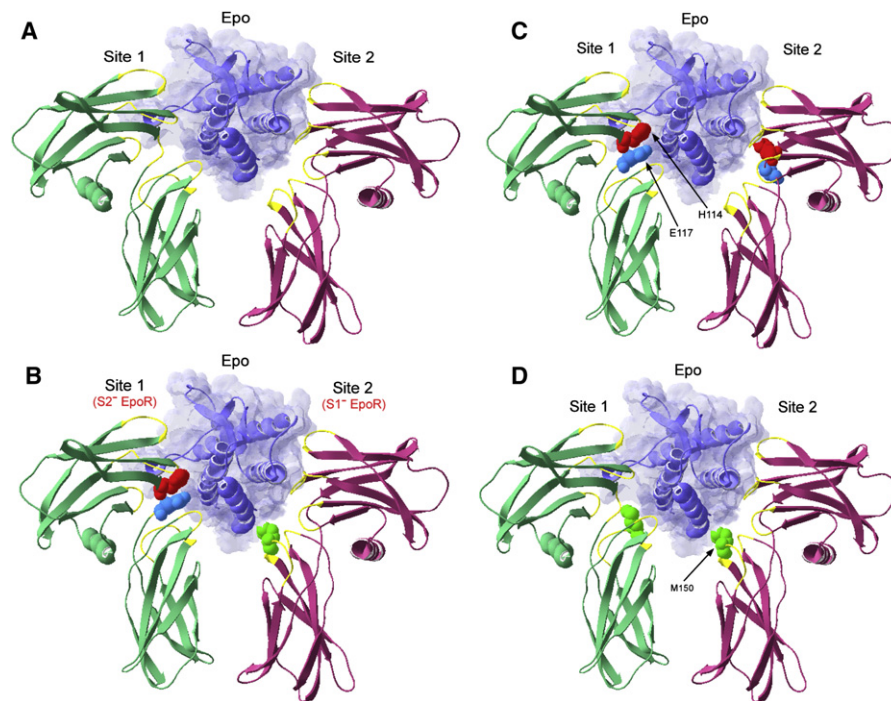
Figure 1. Structures of Epo Complexed with Wild-Type and Mutant EpoR Dimers

(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 (data 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, and 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 His114 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.

understand the role of contributing structural elements in each receptor in initiating JAK2 activation and subsequent signaling. Currently, experimental study 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. Here, we have computationally designed a pair of mutant EpoRs able to bind Epo at only site 1 or site 2. Using this 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 probes as tools for gaining a deeper understanding of other homodimeric cytokine receptors.

RESULTS

Computational Design of EpoR Mutants

To generate a pair of human EpoR's that each bind only to either Epo site 1 or site 2, we computationally analyzed mutations at candidate residues in the EpoR-binding sites. 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). For selective reduction of binding at site 1, EpoR His114 and Glu117 were chosen for analysis due to their direct interaction with Epo at site 1 and lack of direct interaction at site 2 (Figure 1C); also, our previous work showed that mutating His114 could lower Epo-EpoR binding affinity. For selective reduction of binding at site 2, we selected EpoR Met150 because it makes closer contacts at the site 2 interface than it does at site 1 (Figure 1D).

The three selected EpoR residues were each computationally mutated in turn at either the site 1 or site 2 interfaces, and the effect on binding affinity was evaluated, with the goal of identifying mutations that would alter binding-free energy at only one of the two sites. For each candidate residue, mutations to all other amino acids were considered except to cysteine, proline, and glycine. The hierarchical approach of Lippow et al. (2007) was used to calculate binding-free energies of mutants and is discussed further in the Supplemental Data. Briefly, for each mutant, the mutated side chain and surrounding residues were repacked

using a discrete conformational search and a pairwise energy function that utilized the CHARMM22 force field (MacKerell et al., 1998). The binding and bound state energies of the global minimum energy conformation and 29 other low-energy conformations were then reevaluated using an energy function involving continuum electrostatic and surface area contributions.

Computational Analysis of Candidate Site 1 and Site 2 EpoR Mutants

Table S1 shows the computed change in the Epo-EpoR binding-free energy, relative to wild-type EpoR, upon 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 yielded the highest computed disruption in binding affinity (over 3.0 kcal/mol), with the primary mechanism of disruption being electrostatic.

We also considered the effect of mutating both His114 and Glu117 to pairs of residues that were individually promising (i.e., either Lys or Arg at either position), as well as the effect of these four sets of double mutations on receptor binding at site 2. Mutating both His114 and Glu117 to basic residues was predicted to abrogate site 1 binding without greatly affecting site 2 binding (Table S2). Of these four predictions, we chose to experimentally synthesize the H114K / E117K double EpoR mutant.

Table S1 shows the predicted change in Epo-EpoR binding-free energy, relative to wild-type EpoR, upon mutation of Met150 at site 2, leaving site 1 unmutated. 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 (see Table S2).

Validation of Mutant EpoR Candidates in Ba/F3 Cells

Based on our computational predictions, we selected the human EpoR double mutant H114K/E117K (henceforth called 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 expression of either mutant in a cell would not elicit a response to Epo, whereas coexpression of both receptors could lead to a reconstitution of response to Epo, based on the formation of functional receptor heterodimers (Figure 1B). Expression of either mutant alone would result 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 coexpressed, 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 that Ba/F3 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 coexpressing 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⁻ EpoR (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.

Epo Binding Affinity of Mutant Epo Receptors

Analysis of equilibrium binding of ^{125}I-Epo to the surface of Ba/F3 cells expressing wild-type EpoR 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 a K_D of 0.14 nM (95% conf. interval: 0.13, 0.15) (Figure 3A), 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 3A). 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 of 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), these 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.

These results were confirmed and extended by a competition binding assay (Figure 3B) in which ^{125}I-Epo binding to Ba/F3 cells expressing our mutant EpoRs was measured in the presence of increasing concentrations of unlabeled Epo or Epo mutant R103A that is deficient in site 2 binding (Elliott et al., 1997). As determined from the IC_{50} concentration, wild-type Epo binds ~3-fold less avidly to the site 2⁻ EpoR than to the wild-type EpoR (0.27 ± 0.05 nM versus 0.66 ± 0.26 nM), a result consistent with the difference seen in the direct Epo-binding experiment (Figure 3A). Also as expected, the site 1⁻ K45D Epo binds very poorly to either the wild-type or the site 2⁻ EpoR. The key result shows that the site 2⁻ R103A Epo binds with precisely the same affinity to the wild-type EpoR and the presumed site 2 mutant EpoR—8.22 ± 1.86 nM versus 8.15 ± 2.95 nM. Since the site 2⁻ R103A Epo retains normal binding at site 1 (Elliott et al., 1997), the only way the two binding affinities could be the same is if the presumed site 2⁻ EpoR M150E retains normal binding to Epo site 1 and is defective only in binding to Epo site 2.

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 dramatically suppresses growth and signaling responses to Epo but does not affect the ability of the Epo receptors to bind JAK2, traffic normally to the cell surface, or bind Epo (Constantinescu et al., 2001b). Here, we ask whether these switch residues have a greater contribution
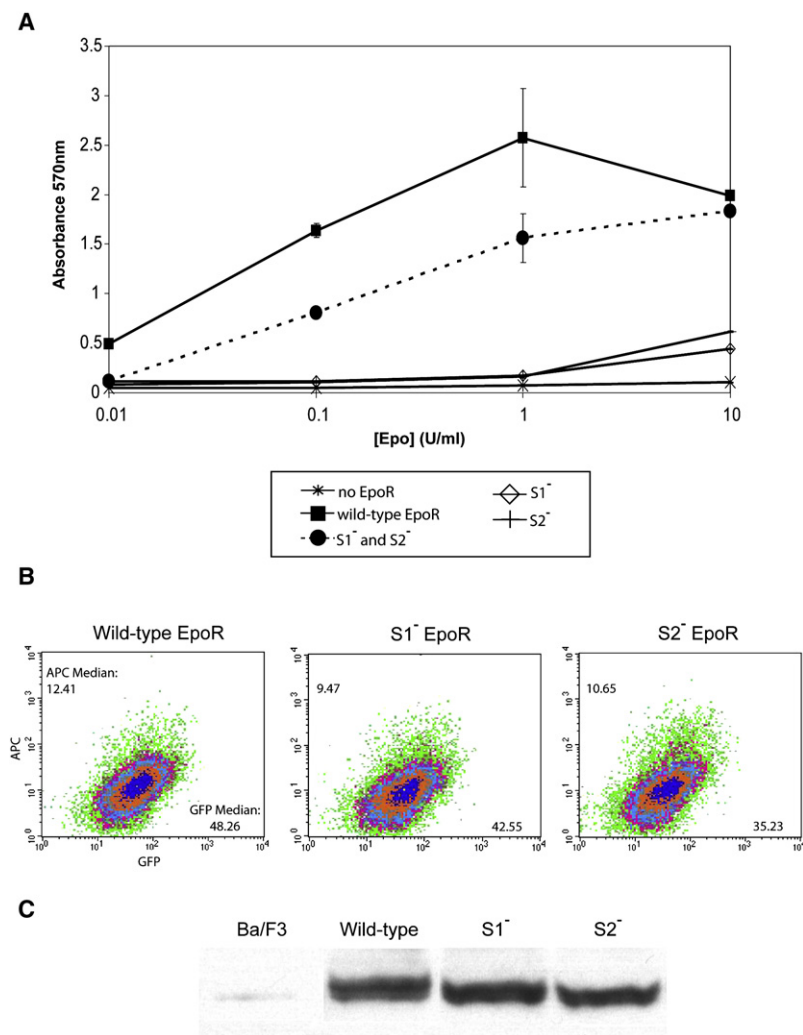
Figure 2. Coexpression of S1⁻ and S2⁻ EpoR Mutants in Ba/F3 Cells Restores Concentration-Dependent Proliferation in Response to Epo

(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 hr 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.

to EpoR activation when they are present on the site 1- or site 2-binding EpoR monomer; 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 and 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.

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- or S2-binding 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 et al., 2001b).

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.

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 et al., 1995b). Activated Stat5 then dimerizes, translocates into the nucleus, and induces expression of genes suppressing apoptosis and promoting proliferation and differentiation (Socolovsky et al.,
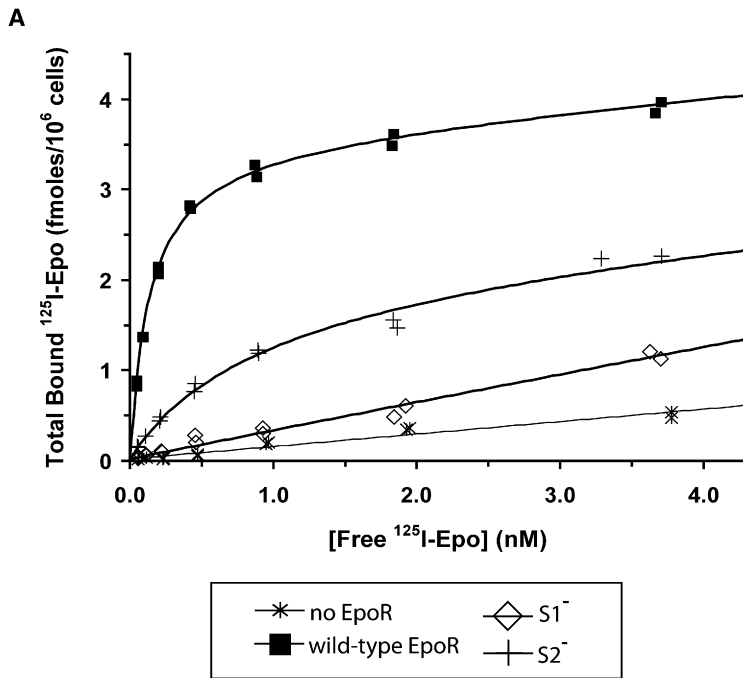
A

Figure 3. Epo Binding by Wild-Type or Mutant EpoRs

(A) Equilibrium binding of ^{125}I-Epo to the surface of parental Ba/F3 cells (no EpoR) or Ba/F3 cells expressing wild-type or S1$^-$ or S2$^-$ mutant EpoR was measured. Binding to parental Ba/F3 cells (no EpoR) represents nonspecific binding. Bound and free ^{125}I-Epo was measured, and binding curves were fitted through the plotted data points as described in the Experimental Procedures. Estimated dissociation constants are as follows: wild-type EpoR, $\sim$0.14 nM; S1$^-$ EpoR, $\sim$77 nM; and S2$^-$ EpoR, $\sim$0.90 nM.

(B) Competition by Epo site 1 and site 2 mutants for binding of ^{125}I-Epo to the surface of Ba/F3 cells expressing wild-type or mutant EpoRs. Binding of 0.5 nM ^{125}I-Epo was measured in the presence of increasing amounts of wild-type Epo, site 1$^-$ K45D Epo, or site 2$^-$ R103A Epo; shown is the IC$_{50}$, the concentration of Epo proteins that inhibited 50% of the specific binding.

B

Cells	Ba/F3-Wild-type EpoR	Ba/F3- Site 2$^-$ EpoR
Competitor protein	IC$_{50}$ (nM) (avg. ± SD)	IC$_{50}$ (nM) (avg. ± SD)
Epo	0.27 ± 0.05 nM	0.66 ± 0.26 nM
Site 1$^-$ K45D Epo	559 ± 301 nM	2827 (1774-3881) nM
Site 2$^-$ R103A Epo	8.22 ± 1.86 nM	8.15 ± 2.95 nM

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 of 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 2-fold higher surface expression of the truncated EpoRs compared to full-length receptors (data not shown). 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 coexpressing 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 coexpressing 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 coexpressing 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).

Importantly, cells expressing S1$^-$ EpoR-H Y343F together with S2$^-$ EpoR-H, and S1$^-$ EpoR-H together with S2$^-$ EpoR-H Y343F, have nearly identical growth responses 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 coexpressing 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.

DISCUSSION

The erythropoietin receptor is a member of a family of cytokine receptors that homodimerize and bind to two discrete sites on a monomeric ligand (Frank, 2002). All contain the same three conserved juxtamembrane hydrophobic amino acids, and all
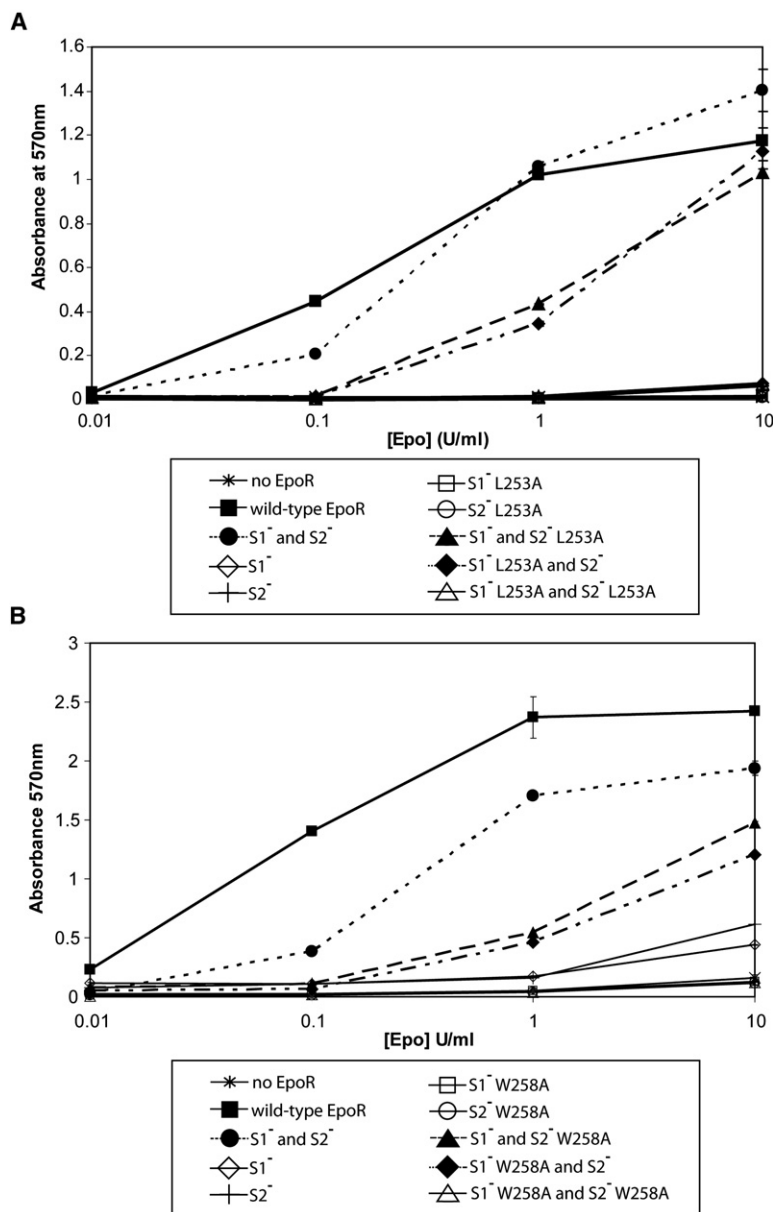
Figure 4. Conserved Hydrophobic Residues Are Needed Only on One Monomer of EpoR to Achieve a Proliferative Response to Epo

(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 hr 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 hr of growth. Data are represented as mean ± standard deviation.

receptors signal equally. 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.

Epo Binding Affinities of Mutant EpoRs

The binding of a single ligand to two identical cytokine receptor monomers was first shown for the interaction of human growth hormone (hGH) with its receptor (hGHbp) (Cunningham et al., 1991); the growth hormone receptor binds with different affinities to two discrete sites— site 1 and site 2— on the monomeric hGh ligand. The fact that a single binding K_D is found for both hGH-hGHbp and Epo-EpoR binding suggests that receptor dimerization is occurring on the cell surface, and 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 and competition binding data is consistent with Philo et al.'s site 1 K_D value because both the wild-type EpoR and the S2$^-$ EpoR (the mutant still able to bind to Epo site 1) have a K_D value for wild-type Epo of ~1 nM. The K_D for the S2$^-$ EpoR is slightly higher than that of the wild-type EpoR; this is because wild-type EpoR can bind Epo at both site 1 and site 2, whereas the S2$^-$ mutant binds only to site 1 on Epo. Our competition studies indicate that the S2$^-$ and wild-type EpoRs bind to Epo Site 1 with the same affinity. In contrast, S1$^-$ EpoR, predicted to be deficient in binding to Epo site 1, as predicted shows little or no specific binding to Epo. The equilibrium binding assay is only useful for measuring the site 1 binding affinity, as Epo site 1 has a 1000-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 is analogous to the G120R hGH mutant that blocks site 2 binding to the growth hormone receptor and that acts as an

activate a Janus kinase and similar intracellular signal transduction pathways; thus, we assume that our results on EpoR activation will apply to these other receptors.

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 deficient in binding to Epo site 1, but not site 2, and another deficient in binding to site 2, but not site 1. Using these 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. Our results suggest that both receptors are competent for signaling, and we suggest that the
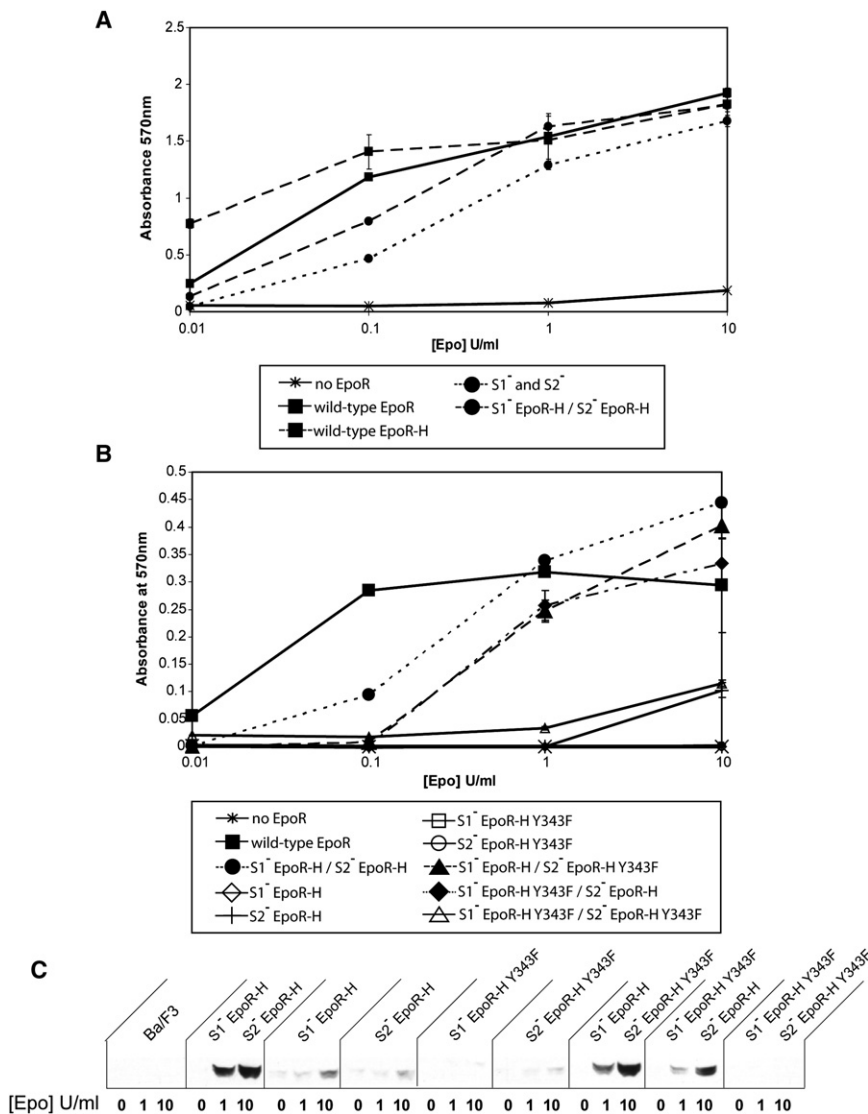
Figure 5. Truncated EpoR with One Tyr343 Is Sufficient for Proliferation in Response to Epo Stimulation

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 hr 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 hr 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 hr at 37°C in RPMI media containing 1% BSA. They were stimulated with Epo at either 0, 1, or 10 units/ml for 10 min 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.

antagonist by binding but preventing receptor dimerization (Fuh et al., 1992).

Functional Activation of the EpoR Heterodimer

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.

These experiments 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. We assume that when S1⁻ EpoR and S2⁻ EpoR are coexpressed, both S1⁻/S1⁻ and S2⁻/S2⁻ homodimers and S1⁻/S2⁻ hetero-

dimers will form in the absence of Epo, but only heterodimers can be activated by Epo stimulation. The formation of nonsignaling homodimers in cells coexpressing both mutant receptors condition likely explains why the "EpoR S1⁻/S2⁻" Ba/F3 strains exhibit a slightly weaker response to Epo when compared to wild-type EpoR.

Hydrophobic Switch Residues and Tyrosine Phosphorylation

We previously proposed that 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 interactions, 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 et al., 2001b), 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, 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, 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. 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 to which it is bound, 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 bind ligand while HER3 lacks kinase activity to phosphorylate cytoplasmic tyrosines. Only when they are coexpressed 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). Structural analysis of the complex between JAK2 and the cytoplasmic domain of cytokine receptors such as the EpoR will be necessary to determine whether there is a *cis* or *trans* relationship between the conserved juxtamembrane hydrophobic residues, the appended JAK2 proteins, and the EpoR tyrosines that become phosphorylated.

EXPERIMENTAL PROCEDURES

Details concerning the preparation of the Epo-EpoR structure and computational analysis of the mutant Epo receptors are in the Supplemental Data.

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 coexpression, 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 hr. 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, intact 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 (50 mM Tris [pH 7.5], 150 mM 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 ~4×10^6 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 hr at 4°C. Bound and free ^{125}I-Epo were separated by centrifuging cells through a layer of FBS. Nonspecific binding was measured with parental Ba/F3 cells which lack detectable Epo-specific binding sites on their surface (Gross

and Lodish, 2006). K_D and B_{max} were determined by fitting the model for a single-class of noncooperative 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 3 days, 100 μl 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 570 nm using a 96-well plate reader.

Phosphorylation Signaling Assays

Ba/F3 cells expressing various hEpoRs were washed and resuspended at 1×10^6 cells/ml in RPMI with 1% BSA, then starved for 4 hr at 37°C. After starvation, cells were stimulated with a range of specified Epo concentrations in RPMI with 1% BSA for 10 min 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 (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, and 5 mM NaF) for SDS-PAGE and western blotting. Phosphorylated Stat5 was detected with polyclonal rabbit anti-phosphoStat5 (pY694) antibody (Cell Signaling Technology, Inc., Danvers, MA).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, two tables, and Supplemental References and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00029-X.

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