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A Diversity of Antibody Epitopes Can Induce Signaling through the Erythropoietin Receptor

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ABSTRACT: Stimulation of red cell production through agonism of the erythropoietin receptor (EpoR) has historically been accomplished through administration of erythropoietin (EPO), the native ligand. The short half-life of EPO has led to the development of a variety of other agonists, including antibodies. It is of considerable interest to understand how these agents might activate the EpoR and whether or not it is important to bind in a manner similar to the native ligand. The binding epitopes of a panel of eight agonistic, single-chain antibody (scFv-Fc) constructs were determined through scanning alanine mutagenesis as well as more limited arginine mutagenesis of the receptor. It was found that while some of these constructs bound to receptor epitopes shared by the ligand, others bound in completely unique ways. The use of a panel of agonists and scanning mutagenesis can define the critical binding regions for signaling; in the case of the EpoR, these regions were remarkably broad.

There has been a great deal of effort devoted to the generation of new erythropoesis stimulating agents (ESAs) with increased half-life, with some approaches already in the clinic (*1*, *2*). Erythropoietin (EPO[1]) is a 30 kDa glycoprotein which stimulates hematopoiesis through the erythropoietin receptor (EpoR) dimer, primarily in the bone marrow. Darbepoetin (Aranesp) is a glycosylated analogue of EPO with two introduced glycosylation sites and an extended half-life. Methoxy polyethylene glycol-epoetin β (Mircera) employs polyethylene glycol to extend the half-life of EPO (*3*). In addition to half-life extension, there is considerable interest in mitigating the risk of immunogenicity to the native ligand through the development of EPO mimetics which are structurally different from EPO. Hematide, "a peptidic erythropoiesis stimulating agent (ESA coupled to polyethylene glycol)" (*4*), has shown efficacy in *in vitro* models and in the clinic. Hemomer is a similar, peptide based approach by Aplagen (http://www.aplagen.com/). A peptide derived from the EPO receptor has been reported to be a potent, noncompetitive agonist (*5*, *6*). A nonpeptide, polymeric compound has also been reported (*7*). The long half-life and favorable clinical track record of antibodies for other disease indications have led to several groups exploring this modality (*8*, *9*).

The EPO receptor (EpoR) is a homodimeric, cell surface receptor with classic features of a cytokine receptor including two fibronectin-like domains with a WSXWS motif near the C-terminal end (*10*). The crystal structures of both native EpoR (*11*) and EpoR bound to EPO (*12*) indicate that the receptor dimer is likely to be preformed (*13*). However, in the absence of ligand, the transmembrane domains are distant, and the cytosolic domains may be too far apart to enable autophosphorylation of JAK2 and signal transduction. Upon binding of a single molecule of EPO, there is a conformational change within the extracellular domain (ECD) of the EpoR which brings the transmembrane regions closer together and allows autophosphorylation and activation of JAK2, initiating the signal transduction cascade (*14*). It is important to consider the orientation of the receptor subunits to the nonbinding agonists for signaling (*15*), and the majority of agonists which operate through this receptor have bound to the same general region of the receptor as EPO (*16*). The ligand binding site has also been elucidated, by both crystallography and mutagenesis studies. The residues F93 and F205 form the basis of the essential hydrophobic interactions with ligand (*11*).

Antibodies which agonize the EpoR are attractive agents for the treatment of anemia due to their long half-life and lack of structural and sequence homology to the native ligand. Previously, it had been shown that agonistic Abs which could induce proliferation of EpoR-expressing cell lines could be generated (*17*−*19*). Recently, the crystal structure of an erythropoiesis-stimulating antibody was released, which revealed that the antibody bound the receptor in a region adjacent to and overlapping that of erythropoietin (*8*).

We sought to generate a panel of single-chain-Fc fusion constructs (single-chain antibodies or scFv-Fcs) (*20*) which agonize the EpoR and to map the regions to which these molecules bind. In order to generate single-chain antibody constructs which signaled through EpoR and agonized through a broad range of modes of binding, a phage display library was panned against a recombinant receptor. The scFvs from the libraries were converted to scFv-Fc proteins and tested for the ability to signal in a functional UT-7 cell assay and for competitiveness with respect to EPO in a FACS binding assay. Although most active constructs competed with EPO, several noncompetitive scFv-Fcs were also found.

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[1]Abbreviations: BSA, bovine serum albumin; EPO, erythropoietin; EpoR, erythropoietin receptor; ECD, extracellular domain; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; scFv-Fc, single-chain variable region fused to antibody Fc; TMB, tetramethyl benzidine; WT, wild-type.

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In order to determine the functional residues involved in binding, alanine scanning of the EpoR was performed. Ninety-five single-residue EpoR mutants were tested for binding to both competitive and noncompetitive scFv-Fcs as well as to EPO itself. Alanine scanning has successfully been used to elucidate critical residues for receptor−ligand interaction (*21*, *22*). Arginine scanning was also carried out on a selection of the 95 original mutants. These methods revealed distinctly different, nonlinear epitopes for the EPO-competitive and noncompetitive antibodies. Importantly, the EPO noncompetitive scFv-Fcs bind to a region which is distal from the EPO binding site, suggesting a different mechanism of action that does not result in interference with natural signaling.

MATERIALS AND METHODS

Phage Display. Single chain antibodies which bound EpoR were isolated from a naïve human single chain antibody fragment (scFv) phage display library containing $> 10^{10}$ clones by standard panning techniques (*23*). The first two rounds of panning were done using biotinylated EpoR extracellular domain (ECD) protein, and the third round of panning was done on a megakaryoblastic cell line, UT-7, expressing native EpoR. Following three rounds of panning, two independent screening procedures were used. The first method employed a phage scFv ELISA to identify clones which bound specifically to recombinant EpoR ECD. The phages which bound were DNA sequenced, and the unique scFv clones were cloned as a fusion to human IgG1-Fc. The second screening method used batch conversion of the scFv inserts (NcoI and NotI restriction fragments) from the third round binding-enriched phage pool to the human IgG1-Fc fusion expression vector (via PciI and NotI sites). Then, 96 × 20 individual transformants were picked, and miniprep DNA was made for transfection to COS-PKB E5 cells in a 96 well plate format. The EpoR agonistic activities were evaluated in a UT-7 cell-based assay using either purified antibodies or the crude mammalian expression supernatants. A minority of constructs which bound the EpoR were agonistic, and none had the potency of EPO as shown in Figure 1. As previous work has shown that the conversion of single chain constructs to antibodies often led to a loss of activity (data not shown), these constructs were kept in an scFv-Fc format.

scFv-Fc proteins were expressed transiently in mammalian COS-1 PKB E5 cells by transient transfection (*24*, *25*). The expressed proteins were purified to greater than 95% purity from the conditioned media using protein A affinity chromatography. Protein identities were verified by N-terminal amino acid sequencing, and concentrations were determined by absorption at 280 nm. Binding to EpoR and competition with EPO was verified by flow cytometry using UT-7 cells.

Cross-Competition of Anti-EpoR scFv-Fc Proteins. Competition studies were performed to determine EpoR binding characteristics of scFv-Fc proteins using a ForteBio Octet instrument (Fortebio, Menlo Park, CA). Three hundred sixty nanomolar biotinylated EpoR in 2% BSA/PBS/0.1% Tween20 was coated onto a streptavidin sensor (Fortebio, Menlo Park, CA) for 15 min and then a new baseline reacquired. Saturating concentrations of individual scFv-Fc protein were then captured by biotinylated EpoR for 15 min, and then 100 nM of the same or another scFv-Fc protein was captured for 15 min.

Mutagenesis of the Erythropoietin Receptor. The crystal structure of the extracellular ligand-binding domain of EpoR complexed to the ligand (PDB ID 1EER (*12*)) was used to design

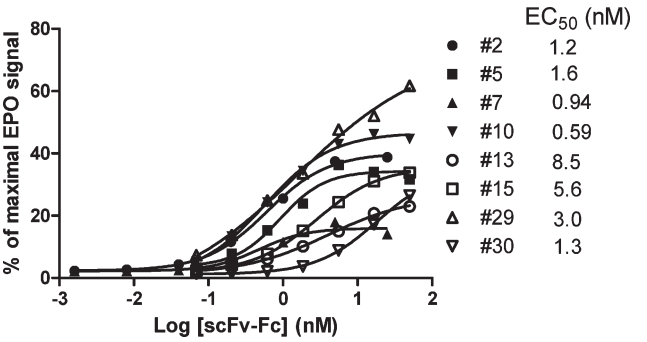

FIGURE 1: scFv-Fc proteins agonize in a UT-7 cell-based proliferation assay. The EC_{50} of each scFv-Fc is listed; in comparison, the EC_{50} of EPO is 19 pM.

a panel of mutants for mapping individual surface residues involved in antibody binding. Structural analysis and image generation were performed using Molecular Operating Environment (Chemical Computing Group, chemcomp.com). An alanine-scanning strategy was primarily pursued for EpoR with additional sparse arginine scanning. The method used to choose residues to mutate involved both computation and interactive structure analysis. The solvent exposures of all residues in the dimer were calculated. Residues with ≥60 Å^2 surface area or with solvent exposure ratios ≥50% were selected for mutation with the exception of glycines with positive Φ angles, prolines in turns, and others based on structural considerations. Solvent exposure ratios, R, were calculated using $R = SE_N/SE_T$, where SE_N is the residue solvent exposure in the native protein structure, and SE_T is the residue solvent exposure in a Gly-X-Gly trimer in which all of the native atom positions are maintained. Further residues were then chosen to fill in surface gaps by examining the structure for residues pointing toward the surface but which were excluded in the solvent exposure calculations. The selected nonalanine residues were mutated to alanine, and those which were alanine in the WT receptor were mutated to serine (Supporting Information). The sequence of the crystal structure differs from human EpoR in that it contains three mutations: N52Q, N164Q, and A211E. While this structure was used for the analysis and the generation of figures in this study, the wild type sequence (accession NP_000112) was used for epitope mapping.

Ninety-five individual alanine or serine mutants were produced, along with the wild-type control. Sense and antisense oligonucleotides containing the mutated residues were synthesized in a 96-well format. Mutagenesis of the WT huEpoR was performed using a QuikChange II kit (Stratagene). All mutants were constructed in a CMV-promoted expression vector (pDC412), and were tagged with 6xHis-AviTag (*26*) on the C-terminus. Mutagenesis reactions and transformations were performed in a 96 well format. 293-6E suspension cells (NRCC) were transiently transfected (*27*). The expression levels and integrity of the recombinant proteins in conditioned media were checked by Western analysis.

ELISA Binding Assay to EpoR Mutants. An ELISA binding assay was used to measure binding of the scFv-Fc proteins to conditioned supernatants containing the mutant protein of interest. One hundred microliters of scFv-Fc proteins at 1 μg/mL in 1× PBS was coated upon a Nunc Maxisorp plate, and incubated at 4 °C overnight. After blocking the wells with 2% BSA/PBS/0.1% Tween20 for 1 h at room temperature, plates were washed three times with PBS/0.1% Tween20. EpoR mutant protein concentrations were normalized on the basis of gel

densitometry relative to the WT protein. The EpoR mutant proteins were serially diluted 3-fold in 0.1% BSA/PBS/0.1% Tween20, which also contained a constant 1:5000 dilution of a-6xHis mAb-HRP (R&D Systems, Minneapolis, MN). The EpoR/a-6xHis mAb mixture was captured for 2 h at room temperature by the scFv-Fc proteins. Tetramethyl benzidine (TMB) was used as substrate and the reaction stopped with phosphoric acid, and the absorption was measured at 450 nm on a plate reader. Binding data were analyzed by nonlinear regression analysis (sigmoidal dose−response and variable slope) to generate EC_{50} values using GraphPad Prism software.

ELISA Binding Assay to EPO. An ELISA binding assay was used to measure the binding of EPO to conditioned supernatants containing the mutant protein of interest. One hundred microliters of biotinylated EPO at 1 μg/mL in 1× PBS was coated upon a NeutrAvidin plate (Pierce Biotechnology, Rockford, IL) for 30 min. After blocking the wells with 2% BSA/PBS/0.1% Tween20 for 1 h at room temperature, plates were washed three times with PBS/0.1% Tween20. EpoR mutant protein concentrations were normalized on the basis of gel densitometry relative to the WT protein. The EpoR mutant proteins were serially diluted 3-fold in 0.1% BSA/PBS/0.1% Tween20, which also contained a constant 1:2000 dilution of a-6xHis mAb-HRP (R&D Systems). The EpoR/a-6xHis mAb mixture was captured for 1.5 h at room temperature. Tetramethyl benzidine (TMB) was used as the substrate and the reaction stopped with phosphoric acid, and the absorption was measured at 450 nm on a plate reader. Binding data were analyzed by nonlinear regression analysis (sigmoidal dose−response and variable slope) to generate EC_{50} values using GraphPad Prism software.

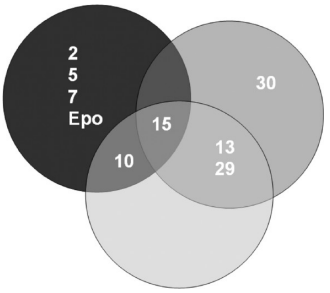

FIGURE 2: Cross-competition was performed to determine which constructs bound to overlapping epitopes on the receptor and which ones competed with EPO. Each circle surrounds a set of competing constructs.

RESULTS

Cross-Competition between scFv-Fc Proteins and Competition with Erythropoietin. In order to map the general epitopes to which these constructs bound, cross-competition experiments were performed both among the constructs and with recombinant human erythropoietin (rHuEPO). UT-7 cell surface binding of clones #2, #5, #7, #10, and #15 was blocked by the addition of an excess amount of rHuEPO. HuEPO did not block the binding of clones #13, #29, and #30. In addition, the scFv-Fcs which competed with huEPO for binding, namely, #2, #5, #7, #10, and #15, also competed with each other. Clone #15 competed with EPO and with all other clones, suggesting that it spans the binding sites of the other two categories of binders. Clone #10 also competed with several of the non-EPO competitive clones. In addition, there was overlap in competition between several of the EPO-competitive and EPO-noncompetitive clones, suggesting that there were subtle differences in binding sites for both categories. Figure 2 shows a diagram of the competition properties of the scFv-Fc proteins.

Construction, Expression, and Characterization of Receptor Mutants. Figure 3 illustrates the expression results and the distribution of the mutant receptor constructs. The average expression level was estimated to be 5 μg/mL. The following mutants were not able to be epitope-mapped due to low expression: E25A, R32A, S54A, K65A, Q71A, R108A, W209A, and W212A. In residues where the mutations were outside of the EPO binding site, receptor mutants were checked for the ability to bind EPO in order to confirm that they were correctly folded. In addition, mutants which did not bind any of the scFv-Fcs were assumed to be misfolded, as competition studies had already shown that there were distinct EPO and non-EPO competing scFv-Fcs. Positions for mutation to arginine were picked upon the basis of the initial mapping analysis with alanine mutants. E34R, E60R, P63R, W64R, T87R, A88R, R99E, A103R, V112R, M150R, H153R, and A166R were mutated, expressed, and analyzed in the same way as the alanine mutants. All of these constructs expressed well.

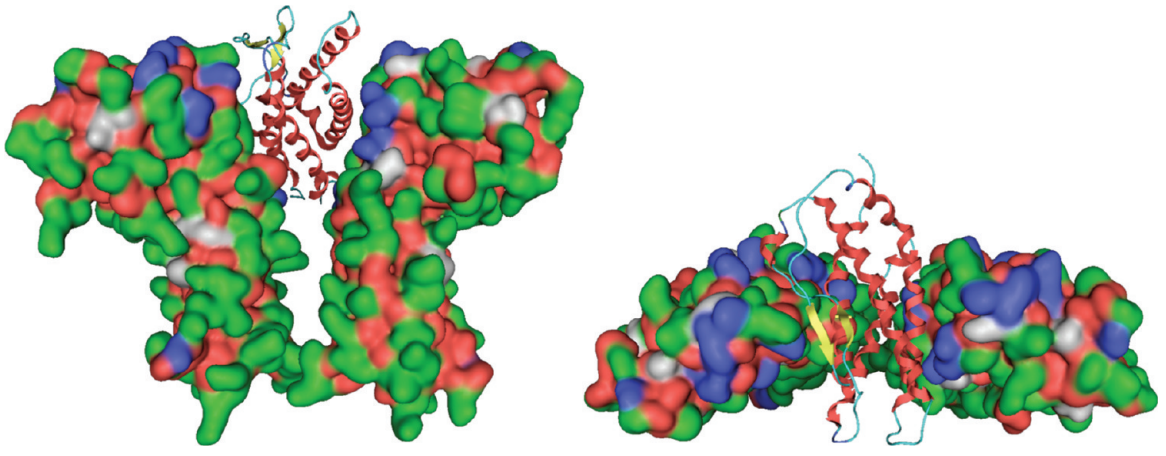

FIGURE 3: Residues selected for mutation. This image of the erythropoietin receptor is based upon the structure (PDB ID 1EER) deposited in the PDB of the ligand-bound extracellular domain. A view from the side is shown on the left and view from above on the right. Shown in green are those residues which were mutated to alanine/serine, in blue are residues that were mutated to both alanine/serine and arginine/glutamate, in gray are residues that were alanine mutants which did not express sufficiently well, and in red are residues that were not mutated. The EPO ligand is shown as a ribbon diagram.

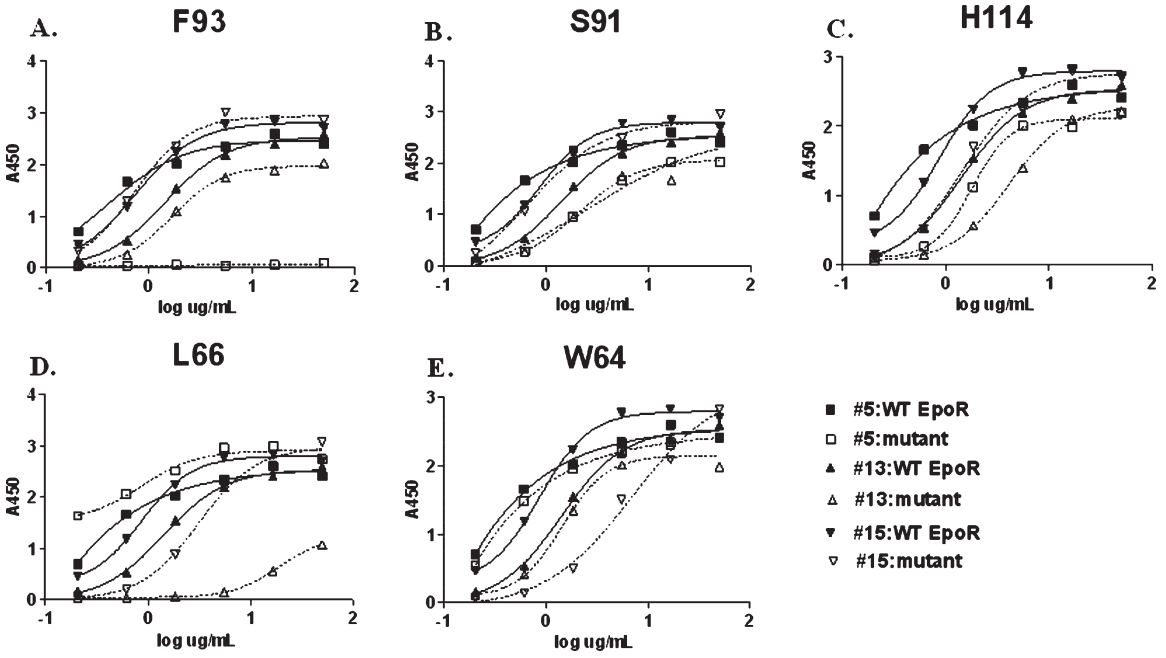

FIGURE 4: Representative data showing the impact of the alanine mutations F93A, S91A, H114A, L66A, and W64A compared to the WT EpoR on the binding of the EPO competitor scFv-Fc #5, the noncompetitor scFv-Fc #13, and scFv-Fc #15, which overlaps #5 and #13. Mutation of the critical contact residue F93 abolishes the binding of scFv-Fc #5 but not the other scFv-Fcs (A). S91A (B) and H114A (C) have a more limited impact on binding. (D) Binding of the noncompetitor scFv-Fc #13 is affected by the L66A mutation. (E) scFv-Fc #15 is affected by the W64A mutation.

Binding of scFv-Fcs to the Mutant Receptor Constructs. Mutations which either abolished binding or decreased binding by 50% relative to WT EC50s were considered part of the epitope. Representative data is shown in Figure 4. The results of binding to the receptor mutants for all of the scFv-Fc constructs are shown in Table 1 and graphically in Figure 6. F208A and P86A were not considered valid epitope hits because neither EPO nor any of the scFv-Fcs of the antibodies bound them, indicating that these mutations probably caused EpoR to misfold. Figures 7 and 8 illustrate the binding of #10, an EPO-competitive scFv-Fc, and #29, a non-EPO-competitive scFv-Fc, respectively. Figure 9 maps out the epitope of scFv-Fc #15, which competed with all other constructs and with EPO. Its epitope can be seen to be between that of scFv-Fc #10 and scFv-Fc #29.

Table 1: Mutants Which Reduced Binding by 50% or More

scFv-Fc	alanine mutants	arginine mutants
#2	F93, H114	E34, E60
#5	S91, F93, H114	E60
#7	F93	E60
#10	E62, F93, M150	A88, M150
#13	V48, E62, L66, R68, H70	
#15	V48, W64, L66, R68, H70	T87
#29	A44, V48, P63, L66, R68, H70	P63, W64, R99E
#30	L66, R99	R99E
EPO	E34, E60, E62, H70, F93, R99, H110, R111, V112, R171	N.D.

DISCUSSION

We discovered that the EpoR can be agonized through a variety of binding epitopes. The epitopes for these scFv-Fc proteins fall into two distinct classes: EPO ligand-competitive scFv-Fc proteins (scFv-Fc #2, #5, #7, #10, and #15) and those which do not compete with EPO (scFv-Fc #30, #13, and #29). These data are consistent with the hypothesis that the non-EPO competitive scFv-Fc proteins agonize EpoR by binding to regions which are distal to the ligand-binding pocket of the dimer. It is somewhat surprising that there did not appear to be a correlation between the ability of an scFv-Fc to compete with EPO and to agonize, as shown in Figure 1.

Alanine scanning did not reveal a large number of residues which were critical for the binding of each antibody. This underscores that alanine mutagenesis is relatively subtle and will primarily reveal side chains which are functionally involved in binding, thus defining the *functional* epitope. The area covered by the antibody, the *structural* epitope, or covered patch will include residues which do not affect binding when mutated to alanine. In order to more fully elucidate the structural epitopes, 11 positions were mutated to arginine, and one WT arginine was mutated to glutamic acid. The arginine side chain is sterically large, effectively blocking antibody binding regardless of whether the wild type residue is necessary for binding the antibody. Arginine mutations have been employed in the past to investigate the binding of thrombin (*28*) and p53 (*29*).

Multiple examples of the effectiveness of arginine mutations can be seen in this study. Figures 7−9 each contain residues, colored blue, which did not have an effect as alanine mutants but did have an effect when mutated to arginine. Figure 5 is a sample of the dose−response data which underlies these figures. The P63R mutation affects the binding of scFv-Fc #29 to a greater extent than does P63A: the arginine mutant abolishes binding (Figure 5C). The mutation of P63 did not cause any change for any of the other scFv-Fcs (e.g., Figure 5A), which is evidence that the protein is essentially well-folded. scFv-Fc #10 is an intermediate case where neither arginine nor alanine substitutions at M150 completely abolished binding, but the arginine replacement had a greater effect (Figure 5B). Again, the mutation of M150 did not significantly alter the binding of any other scFv-Fcs (e.g., Figure 5D); therefore, the receptor is considered to be properly folded. Mutations might be predicted to have the

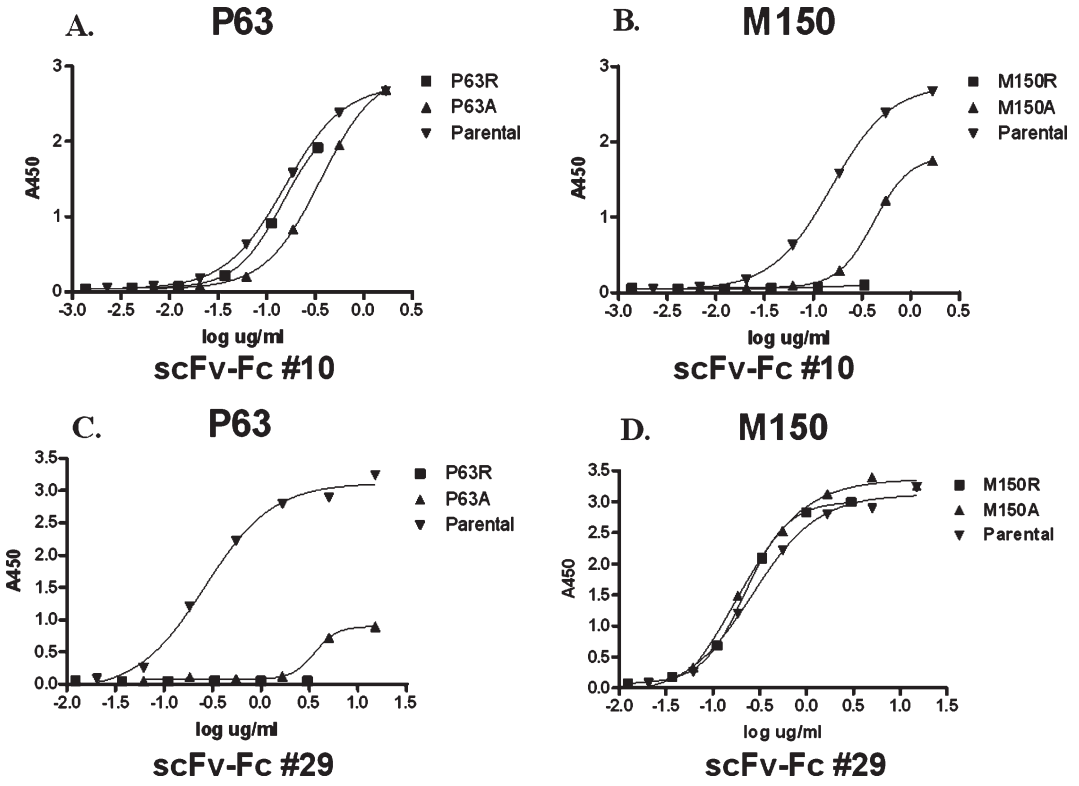

FIGURE 5: Representative data showing the greater impact of arginine mutations relative to alanine mutations. Panels A and B use scFv-Fc #10, which is EPO-competitive, while panels C and D use scFv-Fc #29, which is not EPO-competitive. Both scFv-Fc constructs compete with each other, but mutations at P63 affect #29 much more than #10, while mutations at M150 affect #10 more than #29.

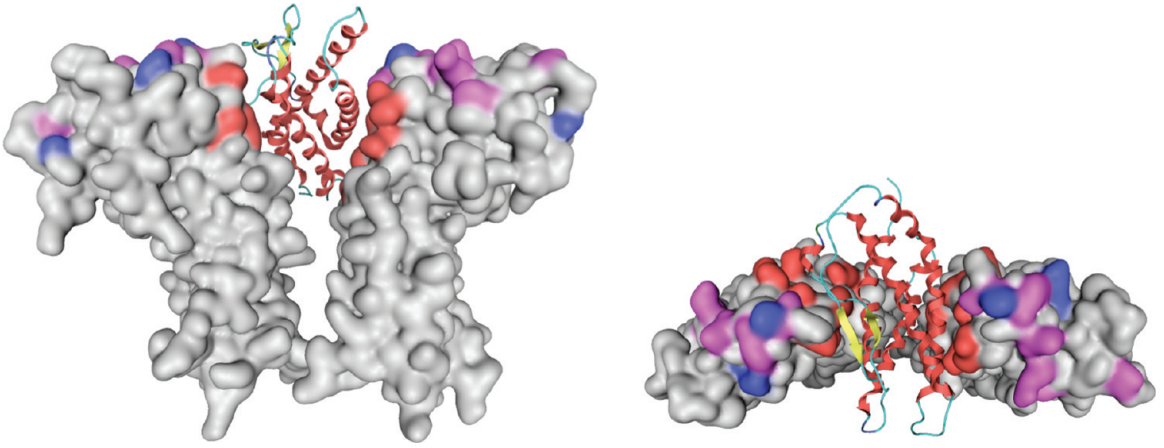

FIGURE 6: All mutations which affected the binding of a scFv-Fc construct are shown here. Red, mutations which affected the binding of EPO-competitive constructs; blue, mutations which affected the binding of EPO noncompetitive constructs; magenta, mutations which affected the binding of both categories of constructs.

greatest impact when they replace a residue with a much different steric bulk or charge. It follows that arginine substitution of a residue with a large side chain might not affect binding as much as alanine substitution would. One case in this set of mutants, W64, bears out this prediction: replacement of the bulky tryptophan residue with alanine had a much more profound effect than did arginine on the binding of scFv-Fc #15. While arginine scanning would have a tendency to flesh out the wider, structural epitope (the bound patch), there may be individual cases where the steric bulk of arginine is tolerated to a greater extent than the small alanine side chain. The use of a panel of substitution residues at each site would help to more completely elucidate the role of that site in binding, but this quickly becomes an enormous set of mutants. Interrogation of such a set has been accomplished elegantly via yeast display with a library of epidermal growth factor mutants, although this kind of approach relies upon statistical oversampling for coverage, and representation of all mutants is not likely (*30*).

scFv-Fcs #2, #5, and #7 are very closely related, sharing identical VH chains and about 2/3 identity in the VL chains (*31*). It is instructive to observe that these also have closely related functional and structural epitopes. The F93A mutation limited the binding of all of these, while structurally neighboring residues S91A and H114A affected the binding of only one and two scFv-Fc proteins, respectively. Structurally adjacent residue E60 did not affect the binding of any of these scFv-Fc proteins when mutated to alanine but effectively eliminated the binding of all three when mutated to arginine. All EPO-competitive scFv-Fc

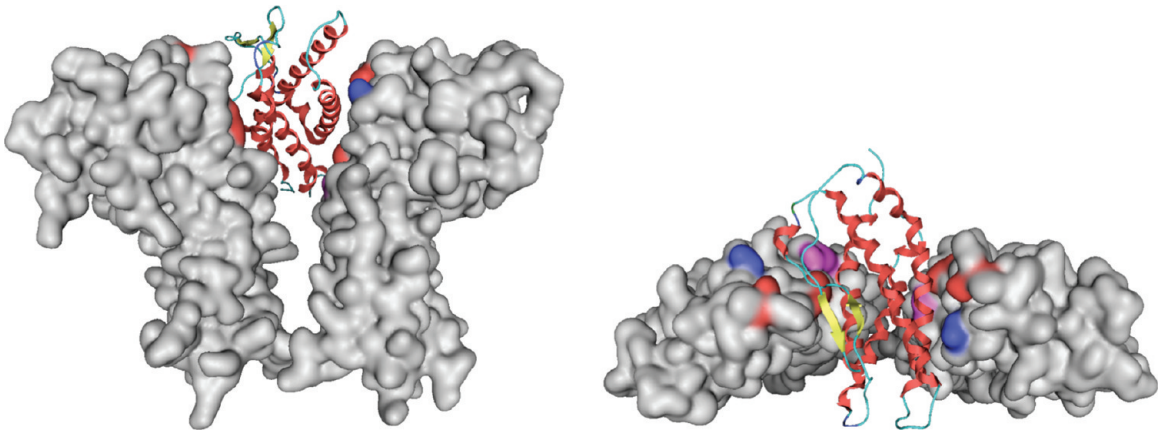

FIGURE 7: Mutations which affected the binding of scFv-Fc #10, an EPO-competitive construct. Red, alanine mutation; blue, arginine mutation only; magenta, both alanine and arginine; gray, no effect or not mutated.

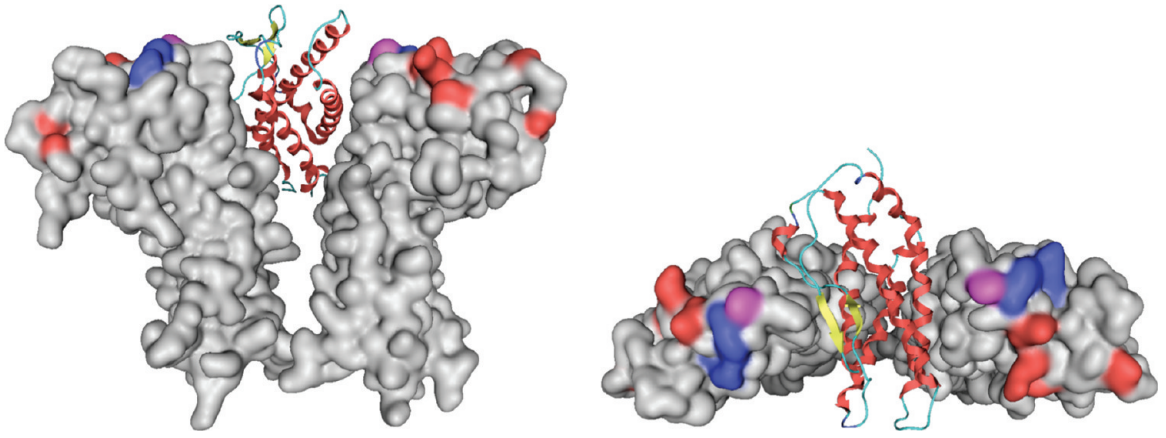

FIGURE 8: Mutations which affected the binding of scFv-Fc #29, a construct which did not compete with EPO. Red, alanine mutation; blue, arginine mutation only; magenta, both alanine and arginine; gray, no effect or not mutated.

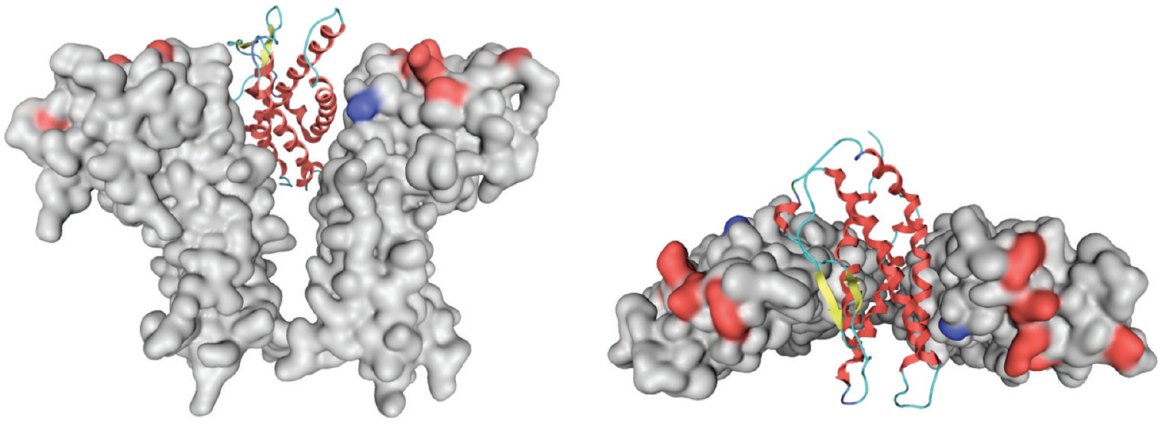

FIGURE 9: Mutations which affected the binding of scFv-Fc #15, a construct which competed with both EPO and all other scFv-Fcs in this study. Red, alanine mutation; blue, arginine mutation only; magenta, both alanine and arginine; gray, no effect or not mutated.

proteins share the epitope F93, which is one of the critical EPO contact residues in the receptor identified in the crystal structure. Shown in Figure 4A−C are representative binding data for F93A, S91A, and H114A, respectively, on the EPO competitor scFv-Fc #5, the noncompetitor #13, and #15 (which competes with both #5 and #13). In addition, binding to scFv-Fc #10 is abolished by the mutation of M150 to either alanine or arginine, another residue in the EPO binding site, illustrating that these scFv-Fc proteins bind to the same region as EPO. As found by others, the M150A mutation did not affect the binding of EPO, emphasizing that the key residues found in a crystal structure may not correlate with solution binding (*32*). F205, a contact residue in the crystal structure, was not part of the panel of mutants assayed in this study as it was not found to have sufficient surface exposure by our criteria.

F93, a critical residue for contact for the ligand (*11*) is notably not part of the epitope of the non-EPO competitive agonists #13 (Figure 4A), #29, and #30. There may be diversity among the epitopes which are capable of signaling through EpoR. Among the scFv-Fc constructs which were not EPO competitive, all shared a sensitivity to L66A as shown in Figure 4D where binding of the noncompetitor #13 is affected by that mutation. Construct

#15 is a special case, as it occupies a binding site close enough to the binding regions of both the competitive, noncompetitive, and ligand binding sites to compete with them all. V48 and H70, hits for the noncompetitive constructs #13 and #29, were also hits for #15, but #15 did not share hits with the EPO competitive constructs, including F99A. It should be remembered that these constructs have considerable steric bulk relative to the receptor and that there is an inherently limited capacity to simultaneously accommodate the binding of multiple scFv-Fcs. All things considered, the multiple activating epitopes described here point to a diversity of potential modes through which the EpoR can be activated.

The recently released crystal structure of an erythropoiesis-stimulating antibody Fab, ABT007 (PDB ID 2JIX), revealed 14 individual molecular interactions which were determined by the authors to be important for binding (*8*). Eight of the 10 residues which were found to have productive interactions in the crystal structure were also mutated in this study. Only H114 was shared by any of the EPO-competitive scFv-Fc proteins tested here, while residues R99 and W64 (Figure 4E) were shared with several of the noncompetitive scFv-Fc proteins. This small overlap again suggests that overall EpoR conformation, rather than the specific interactions, is important for productive EpoR signaling.

The structure of the native receptor in the absence of ligand (*11*) indicates that a large, scissoring rotation of the preformed receptor dimer must take place for signaling to occur. As such, the binding of a dimeric scFv to the membrane distal domains of the two receptor halves could induce the same kind of scissoring rotation which is induced by EPO. Interestingly, the peptide agonist described by Naranda et al. (*5*, *6*) was derived from the EpoR in a region quite close to the membrane, from the location where the two receptor monomers interact. Presumably, the peptide interacts with the receptor in this region. This is in contrast to the scFv-Fc constructs which all bound regions distal from the membrane, a result which may be due to the relative steric bulk of the scFv-Fc constructs, the distance between the two arms of the scFv-Fc, or, more trivially, on the panning method employed to find them. That all of these diverse epitopes can be utilized to productively signal though the receptor is remarkable.

There are a large number of methods which have been employed for mapping the epitopes of antibodies. Peptide-based methods, which rely upon the binding of antibodies to short fragments of their antigens, have been used extensively but are not effective when the binding of the antibody is context or conformation dependent. Crystallography provides an extraordinarily detailed image of the bound epitope, but not every antibody/antigen pair will provide diffracting crystals, and it is typically a highly consumptive method, both in effort and required protein. Alanine scanning is still a mainstay of epitope mapping, but as was shown here, the subtlety of the effect of an alanine mutation requires a large set of mutants and does not disclose the structural epitope. Some efforts have focused on increasing the impact of an introduced mutation by mutating a residue to cysteine and then either chemically conjugating a bulky residue (*33*) or masking the mutated region by binding the target to a solid support (*34*). Epitope mapping methods which rely upon mass spectrometry, such as deuterium exchange, show enormous promise (*35*), but these also are very sensitive to slight differences in methodology (e.g., time, temperature, and pH) and may therefore suffer from reproducibility issues (*36*).

We have demonstrated that alanine and arginine mutagenesis can be used for the facile determination of important interactions for large numbers of antibodies, bypassing the necessity of exhaustively cocrystallizing individual antigen−antibody pairs. However, mutants which perturb the structure or greatly diminish expression leave gaps in the coverage. Only 11 out of 95 residues mutated were misfolded or expressed at levels too low to be used in the binding assay, though indicating that adequate coverage is achieved by this technique.

These observations have utility beyond the characterization of these scFv-Fc constructs. There appears to be a patch at the top of the receptor which can be utilized for scFv-Fc signaling without disrupting endogenous erythropoietin signaling. Negative selection against mutants in this region could be used to enable high-throughput techniques such as phage panning and selection without the need to laboriously test thousands of clones in a bioassay. Importantly, this kind of epitope mapping could be generalized to other receptor systems where ligand-independent agonism is the goal.

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SUPPORTING INFORMATION AVAILABLE

Complete list of the mutants generated. This material is available free of charge via the Internet at http://pubs.acs.org.

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