Dimerization of the Erythropoietin Receptor Transmembrane Domain in Micelles

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Erythropoietin receptor (EpoR) homodimerization is an initial regulatory step in erythrocyte formation. Receptor dimers form before ligand binding, suggesting that association between receptor proteins is dependent on the receptor itself. EpoR dimerization is an essential step in erythropoiesis, and misregulation of this dimerization has been implicated in several disease states, including multi-lineage leukemias; nevertheless, how EpoR regulates its own dimerization is unclear. In vivo experiments suggest the single-pass transmembrane helix is the strongest candidate for driving ligand-independent association. To address the self-association potential of this transmembrane segment, we studied its interaction energetics in micelles by utilizing a previously successful Staphylococcal nuclease (SN-EpoR TM) fusion protein. This fusion protein strategy allows expression of the EpoR transmembrane domain in Escherichia coli independent of the other EpoR domains. Sedimentation equilibrium analytical ultracentrifugation of the detergent-solubilized SN-EpoR TM demonstrated that the murine EpoR transmembrane domain self-associates to form dimers. Although this interaction is not as stable as the dimerization of the well-studied glycophorin A transmembrane dimer, the murine EpoR transmembrane domain dimer is more stable than the interactions of the colon carcinoma kinase 4 transmembrane domain. The same experiments with the human EpoR transmembrane domain, which differs from the mouse sequence by only three residues, revealed a less favorable interaction than that of the murine sequence and is only slightly more favorable than that expected for non-preferential binding. These results suggest that the mouse and human receptor proteins may differ in the roles they play in signaling.

Keywords: erythropoietin receptor; transmembrane dimer; membrane protein interaction; thermodynamics; analytical ultracentrifugation

Introduction

The erythropoietin (Epo) receptor and its ligand are crucial for effective proliferation and differentiation of mammalian pluripotent hematopoietic stem cells and survival of erythrocyte progenitors. A normal human erythrocyte lifespan is approximately 120 days, and the number of circulating erythrocytes is maintained by replacing $10^{11}$ erythrocytes each day. Therefore, tight regulation of erythropoiesis is essential for normal biological function: too much activity, caused by constitutive receptor expression, induces multi-lineage leukemias; too little activity can result in anemia. Regulation of erythrocyte formation occurs at several locations in erythropoiesis. Transcriptional activation of target genes in hematopoietic stem cells results from a signaling cascade initiated by Janus kinase 2 protein phosphorylation of multiple tyrosine residues on the Epo receptor (EpoR) intracellular domains. These two Janus kinase 2 proteins are cytoplasmically associated with EpoR and must come within proximity of each other for this initial signaling step to occur. This is achieved when Epo

Abbreviations used: C14SB, 3-(N,N-dimethylmyristyl-ammonio)propanesulfonate; Epo, erythropoietin; EpoR, erythropoietin receptor; EpoRh, human EpoR; EpoRm, mouse EpoR; GpA, glycophorin A; SN, Staphylococcal nuclease; SRV, square root of variance; TM, transmembrane.

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doi:10.1016/j.jmb.2006.11.035
binds to the extracellular domain of pre-formed inactive receptor dimers that causes a conformational change, conferred through the transmembrane helices, to the intracellular signaling domain and allows close contact of the Janus kinase 2 proteins.4,5

This complex pathway requires multiple protein interactions and possesses several natural feedback loops that help to control normal differentiation.4,5 The first regulatory step of this pathway, however, is EpoR dimerization, which initially determines the number of receptors available for ligand binding. The details of how EpoR regulates its homodimerization are not known. EpoR is a 66 kDa integral membrane protein with extracellular and intracellular domains, as well as a single-pass helical transmembrane domain that transverses the phospholipid bilayer. Several residues in the extracellular domain have implicated this region of the protein in driving dimerization. Although a crystal structure also supports this hypothesis,6 in vivo immunofluorescence colocalization studies suggest that it is the transmembrane domain that encodes the molecular information important for dimerization in cells, not the extracellular domain.7 In these colocalization studies, chimeric receptors generated by domain-swapping between murine Epo receptor and the non-associating rabbit prolactin receptor were expressed in vivo as full-length proteins. Using immunofluorescence colocalization to visualize ligand-independent association, the resulting chimeras showed a propensity for colocalization comparable to wild-type EpoR only when they contained the EpoR transmembrane domain.7 In contrast, chimeric receptors containing either the intracellular or extracellular domains of EpoR showed a 30% decrease in colocalization compared to that of the wild-type receptor. Thus, only the transmembrane helical region of EpoR is sufficient to maintain dimerization of the full-length protein while the intracellular and extracellular regions alone could not. In an independent study, Kubatzky et al. isolated the transmembrane (TM) domains of both the mouse and human EpoR proteins and tested their ability to self-associate in membranes using the ToxR interaction screen.8 In this study, both the mouse and human Epo receptor transmembrane domains showed propensities for dimerization that were almost as strong as that observed for the glycoporphin A transmembrane dimer, the positive experimental control. Interestingly, the activity of the mouse transmembrane domain was slightly higher than that of the human TM domain, suggesting that slight differences in propensities for self-association may exist between the two homologues.

Studies with EpoR-null mice containing a transgenic human Epo receptor also seem to support the hypothesis that slight differences exist in receptor propensity between the two species. Mice lacking endogenous Epo receptor exhibit severe anemia and die at embryonic day 13.5.9 This embryonic lethal phenotype can be rescued by introducing a transgenic human EpoR, and these mice survive well into adulthood. However, while the rescue of EpoR-null mice by the human Epo receptor allows the mice to mature into adulthood, these transgenic mice were slightly anemic compared to wild-type littermates, suggesting that restoration of normal erythropoiesis may be incomplete.10 An independent study determined that a human EpoR transgene had expression levels comparable to endogenous mouse EpoR at all phases of development.5,11 Therefore, EpoR-null mice with the human transgene should have the same number of receptors as their wild-type littermates. Additionally, there was no apparent species difference in Epo stimulation of the transgenic receptor. Thus, something else must account for the erythrocyte count in rescued mice. One hypothesis is that this slight difference may result from variations in receptor signal regulation between the mouse and human homologue.

Despite the available cellular studies, very little is known about how EpoR regulates its dimerization. First, does the transmembrane domain drive receptor self-association; and second, can we determine differences between mouse and human receptor regulation? To address these questions, we carried out a thermodynamic study using sedimentation equilibrium analytical ultracentrifugation to determine the propensities of the transmembrane domains of both the human and mouse Epo receptors to self-associate.

**Results**

The mouse and human EpoR TM domains show a propensity to self-associate

To explore their propensities for self-association, the transmembrane domains of both the human and mouse Epo receptors were isolated from the remainder of the full-length receptors by subcloning as C-terminal fusion proteins behind the soluble, monomeric protein Staphylococcal nuclease (SN). This chimeric construct facilitates expression and purification of these TM domains in *E. coli* and analogous SN-TM fusion proteins have been used to study the self-association of a wide variety of transmembrane helices, including glycoporphin A (GpA), colon carcinoma kinase 4 (CCK4), as well as BNIP-3, erbB1-4, Vpr1, Vpx2, Gag and phospholamban.12–18 Because SN shows no detectable propensity to self-associate in the absence of a dimerizing C-terminal region,19 this expression strategy has the advantage of providing a method of untangling the thermodynamic influences of the TM from that of the remaining domains in the full-length receptor protein. Most notably, this fusion protein architecture has been especially instrumental in dissecting the sequence dependence of the stability of glycoporphin A dimerization.5,20,21 **Figure 1** shows the amino acid sequences of the TM segments for both the human and mouse EpoR constructs used in the studies discussed here. These sequences were
brane domains. The advantage of using this method is that the molecular mass can be determined directly; in the case of extremely weak or no interaction, sedimentation equilibrium will still result in a positive experimental finding, since it will report the monomeric molecular masses. The radial distribution profiles of nine equilibrium data sets (three initial protein concentrations at three speeds) were globally fit using a non-linear, least-squares curve-fitting procedure provided by the Windows version of NONLIN.24 Fitting the data to a single ideal species model provides an estimate of the effective molecular mass (\( \sigma \))\(^2\), of the species in solution. These values were determined at two concentrations of detergent micelles for both human and mouse SN-EpoR TM. A comparison of the experimental \( \sigma \) values to the theoretical values calculated at 20,000 rpm and 25 °C for the monomeric proteins results in a \( \sigma \) ratio greater than that expected for a monomer, suggesting that both the mouse and human TM domain either form oligomers or aggregates. This \( \sigma \) ratio was found to be sensitive to the aqueous concentration of detergent micelles and decreased upon dilution of the protein into higher concentrations of micelles, which indicates a reversible association of both receptor TM domains within the micellar phase. In addition, a comparison of these \( \sigma \) ratios at the same concentration of detergent micelles consistently revealed that the mouse EpoR TM domain showed a greater tendency towards self-association than the human EpoR TM sequence. This observation mirrors the trends of increased association propensities for the mouse sequence that have been observed in chimeric receptor constructs in whole-cell experiments.7

To exclude other models, we fit the data to monomer-trimer, monomer-dimer-trimer and monomer-dimer-tetramer models. Because the monomer-dimer-trimer and monomer-dimer-tetramer fits failed to converge, statistics from these fits are excluded from Table 1. As expected from the original effective molecular mass fits discussed above, the sedimentation equilibrium profiles could not be well described by a single ideal fit; inclusion of a non-ideality factor, \( B \), did not improve the fit. The square-root of variance values were higher and the residuals of the graphical fits were not distributed randomly about zero (data not shown). Surprisingly, our analysis of the data using a monomer-trimer model resulted in slightly better fit statistics in three of four cases. Examination of the residuals of the fits for both the monomer-dimer and monomer-trimer models does not allow an unambiguous distinction between these two reaction schemes because the residuals are random and clustered tightly about zero under both experimental conditions. The F-statistics for the monomer-dimer and monomer-trimer fits of the mouse and human SN-fusion protein in both 33 mM and 23 mM C14SB were calculated to determine quantitatively if the fits were significantly different from each other. This analysis could not detect a significant difference between the fits and did not, therefore, rationalize rejecting either model. This is not surprising, since our data for both constructs suggest the presence of only a small population of dimer, and simulated data for a weak monomer-dimer association reaction can be fit equally well by a monomer-trimer model (data not shown).

![Table 1](image)
Cross-linking demonstrates EpoR TM monomers and dimers in solution

The slight statistical preference for a monomer-trimer fit from sedimentation equilibrium data was an unanticipated result in light of the extensive biological data indicating that the EpoR populates dimers and not trimers. We therefore carried out glutaraldehyde cross-linking studies to obtain independent evidence of the stoichiometry of the self-associated species in our experiments. While glutaraldehyde cross-linking is not an equilibrium method, it can provide an indication of the preferred species in solution; in this case, we used it to determine whether dimers or trimers, or both, were present in sedimentation equilibrium samples. Figure 3(a) and (b) show the SDS-PAGE analysis of a cross-linking time-course. We carried out these experiments using protein samples derived from the same preparation that was analyzed by sedimentation equilibrium. In both cases, the cross-linking shows a prominent monomer band migrating at 21 kDa and a visible dimer band at 42 kDa that accumulates over time. At long time-points, we detected high molecular mass species that were not able to enter the gel. However, we did not observe these species in the sedimentation equilibrium experiments. This suggests that these high molecular mass species are stabilized by cross-linking and do not represent a stable equilibrium population. None of our cross-linking experiments ever indicated the...
presence of a stable trimeric form for either the human or the mouse TM domains. Thus, the cross-linking experiments provide a justification for interpreting the sedimentation equilibrium fits using the monomer-dimer reaction scheme.

The mouse TM domain dimerizes more favorably than the human TM sequence

Using the monomer-dimer reaction scheme, estimates for the apparent free energy change can be calculated using the equilibrium constants from the best-fit values returned in the monomer-dimer sedimentation equilibrium fits. The apparent free energies of association of the mouse and human sequences in 33 mM C14SB were found to be \(-5.9(\pm 0.1)\) kcal mol\(^{-1}\) and \(-4.8(\pm 0.3)\) kcal mol\(^{-1}\), respectively, at 25 °C. This finding supports the initial evidence that the mouse TM domain has a greater relative tendency to self-associate. This is best illustrated in Figure 4 by comparing the distribution of species for both the mouse and human EpoR TM domains where the mouse TM domain shows a population of dimer at a lower mole fraction than that for the human TM.

As expected for a reversible association occurring within the micellar phase of the solution, the apparent free energies of association depend on the total amount of micellar detergent available and become more favorable when it is lowered. In 23 mM C14SB the association free energies equal \(-6.5(\pm 0.4)\) and \(-5.1(\pm 0.2)\) kcal mol\(^{-1}\) for the mouse and human TM domains, respectively; this enhancement is due to a concentration of the protein in the detergent micellar phase. This concentration effect can be taken into account by converting the apparent free energy values into standard free energy values using an ideal-dilute assumption for the protein behavior in the micellar phase. When this is accounted for, the standard state free energies of dimerization were found to be \(-3.7(\pm 0.1)\) kcal mol\(^{-1}\) and \(-4.3(\pm 0.4)\) kcal mol\(^{-1}\) for the mouse TM domains in 33 mM and 23 mM C14SB, respectively, and \(-2.8(\pm 0.3)\) kcal mol\(^{-1}\) and \(-3.0(\pm 0.2)\) kcal mol\(^{-1}\) for the human TM domains in 33 mM and 23 mM C14SB, respectively.

Discussion

Mouse and human Epo receptor transmembrane domains drive dimerization weakly in C14SB detergent micelles

Using sedimentation equilibrium, we have shown that the mouse and human Epo receptor transmembrane domains both dimerize in the context of the SN-fusion protein constructs described. This result agrees with both the \(\text{in vivo}\) immunofluorescence colocalization and ToxR studies in bacterial cells, indicating that the transmembrane domain is sufficient for dimerization in the absence of both the extracellular and the intracellular receptor domains.

The availability of these thermodynamic measurements allows a comparison of the self-association propensity of the EpoR transmembrane sequences to those known for the transmembrane domain of GpA and CCK4, which were carried out in the same hydrophobic environment. The distribution of the dimeric species for each of these proteins is shown in Figure 4. GpA, a model for helical transmembrane dimerization, shows a strong propensity for self-association and is commonly used as a positive control for self-association studies in bacterial genetic assays; its stability is reflected in the low mole fraction protein concentration where the GpA transmembrane domain begins to populate dimers. In contrast, a stochastic model for non-preferential binding can explain the weak association of the human CCK4 oncogene. This model describes the random occurrence of two molecules in the same micelle, which has a non-zero probability as the protein to detergent ratio increases. This stochastic dimerization can be described by a standard free energy of \(-2.4\) kcal mol\(^{-1}\), thus representing the upper limit for thermodynamically meaningful self-

![Figure 4](image-url) A comparison of the dimer species distribution of both SN-EpoR TM domains to the GpA and CCK4 SN fusion proteins. The percentage dimer is shown as a function of the mole fraction protein. The distributions were calculated using experimentally determined \(\Delta G^o\) values.
association. Similarly, the transmembrane domains of the human erbB transmembrane domains were found to associate only slightly more favorably than this stochastic limit, although the quantitative analysis of these tyrosine kinase receptors was carried out in a different detergent micelle environment. For this reason, their distributions are not included in Figure 4. Notably, both the mouse and human EpoR TM domains dimerize with a more favorable free energy than that expected for non-preferential binding. This is illustrated in Figure 4, in which both the mouse and human Epo receptor transmembrane domains show a population of dimer at a lower mole fraction protein than the CCK4 TM domain. By comparing the experimental standard state free energy values for the EpoR constructs to that of the stochastic model, thermodynamically meaningful free energies of association of ∼1.3 kcal mol⁻¹ and 0.4 kcal mol⁻¹ can be calculated for the mouse and human sequences, respectively, suggesting there is specificity in the sequence recognition for these two transmembrane interactions. In a biological context, it makes sense that the EpoR domains would have self-association propensities between those observed for GpA and CCK4, since normal receptor regulation would require an association that was not too strong or too weak. Under these conditions, erythrocyte differentiation could be controlled tightly within the narrow range of conditions necessary for normal function. Moreover, unlike the CCK4 and erbB tyrosine kinase receptors, the Epo receptor is pre-dimerized before ligand binding, and the enhanced transmembrane interactions we observe for both the mouse and human EpoR TM sequences may reflect a subtle but functionally important difference in how receptor stability is encoded.

Interestingly, the murine EpoR transmembrane domain has a stronger association than its human homologue, reflecting the same trends observed in vivo as previously discussed. The mouse and human Epo receptor transmembrane domains used in this study differ only in three amino acids, noted by arrows in Figure 1, yet we still observe almost 1 kcal mol⁻¹ greater dimerization propensity for the mouse sequence. There are several possible explanations for this difference. One possibility is that the leucine zipper motif proposed as the driving force for EpoR self-assembly is disrupted in the human domain. The leucine zipper is a dimerization motif characterized by interacting residues forming heptad repeats (abcdefg), where residues at the a and d positions establish the interacting interface. Most often, these positions are held by leucine, isoleucine, valine, methionine and phenylalanine. The sequence motif for EpoR proposed by Gureka et al. contains residues 232, 236, 239, 240, 244, 246 and 247 as numbered in Figure 1. Both L236 and L239 are leucine in the mouse transmembrane domain but are substituted by valine in the human sequence. Doura et al. showed a 2 kcal mol⁻¹ loss in free energy of association when Leu75 was substituted by valine in the GpA TM dimer. Thus, it is quite reasonable that, in the context of the EpoR transmembrane domain, substitution of these two leucine residues may be responsible for the observed loss in free energy of association between the mouse and human sequences. Another possibility is that this difference in transmembrane domain self-association may simply be due to differences in packing effects or van der Waals interactions between the two sequences rather than to a specific motif. Studies of other transmembrane domains, including glycophorin A, have provided strong evidence for the hypothesis that tight packing can provide a dominant force in thermodynamic stability. Therefore, it is possible that these three substitutions alter the packing interface of the Epo receptor sufficiently to result in a 1 kcal mol⁻¹ loss in free energy of the human transmembrane domain compared to that of the mouse sequence.

**Differences in mouse and human Epo receptor TM dimerization may reflect a trend in erythropoiesis regulation**

Our data reveal a quantitative difference in the self-association propensities for the human and mouse EpoR transmembrane domains that is consistent with those shown in previous cellular studies. We postulate that this property may reflect an overall trend in Epo receptor regulation. The fact that the mouse receptor TM dimerizes more strongly than its human counterpart suggests that, at equivalent expression levels, there will be a higher population of dimeric EpoR receptors in murine stem cells as compared to humans. Epo will bind to the available dimeric receptors and trigger a signaling cascade resulting in erythrocyte production. In mice, more receptors would be available for ligand binding; therefore, more signaling and more erythrocytes would be produced. This increase in erythropoietic activity would be necessary under normal maintenance conditions if the life-span of murine erythrocytes was much shorter than that of humans, and in fact, this is the case. Normal human erythrocytes have a life-span of 120 days, while those of mice survive only 55 days before requiring replacement. Therefore, it is reasonable that the murine erythropoietin receptor, which is crucial for erythrocyte formation, would be more active than that of the human. This would also explain why the EpoR-null mice rescued by the transgenic human Epo receptor are able to survive but are still slightly anemic, despite comparable amounts of receptor expression. Although the human receptor is sufficiently similar to the mouse receptor to recognize the native ligand and competently induce a signaling cascade, a lower concentration of receptor dimers would be available to bind Epo. Under these conditions, the subsequent amount of erythrocytes produced would be less than that of the wild-type, resulting in slightly anemic mice.
Materials and Methods

Cloning and expression

Nucleotide primers for both the sense and antisense sequences of the mouse and human EpoR transmembrane sequences were purchased from IDT, Inc. and were designed to encode the amino acid sequences of the respective transmembrane domains. The coding frames for the amino acid sequences were followed by a stop codon, and the primers contained unique Xmal and BamHI restriction sites flanking the N-terminal and C-terminal ends of the sequences to facilitate cloning into the pET11A-SmaGpa99 vector in place of the glycophorin A transmembrane domain. The corresponding amino acid sequences that were expressed as C-terminal fusion proteins with Staphylococcal nuclease (SN) are given in Table 1. The resulting mouse and human SN fusion proteins were transformed into E.coli strain hms(DE3) and expressed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Both the SN-human EpoR (EpoRh) and SN-mouse EpoR (EpoRm) proteins were purified following the protocol published for SN-BNIP3.

Sedimentation equilibrium analytical ultracentrifugation

Immediately before sedimentation equilibrium analysis, purified SN-EpoR TM proteins were exchanged by ion-exchange chromatography into C14SB detergent micelles as described. Sedimentation equilibrium experiments were done with a Beckman XL-A analytical ultracentrifuge in six-sector cells according to previously published protocols. The absorbance at 280 nm was measured for three initial protein concentrations (0.3 mg/ml, 0.6 mg/ml, and 0.9 mg/ml) and three rotor speeds (20,000 rpm, 24,500 rpm and 30,000 rpm). WINMATCH was used to verify that equilibrium was reached at each speed.

Sedimentation equilibrium profiles were analyzed using the Windows version of NONLIN. The monomer molecular mass, \( M \), and the partial specific volumes \( \bar{v} \), of the human and mouse constructs were calculated from the amino acid sequences using SEDNTERP, which was also calculated using the standard Gibbs free energy equation:

\[
\Delta G_{\text{app}} = -RT \ln K_{\text{app}}
\]

The standard state free energy change was calculated using the apparent value under the ideal-dilute assumption using the following equation:

\[
\Delta G = \Delta G_{\text{app}} - RT \ln [\text{micellarDet}]_w
\]

where [micellarDet]_w is the concentration of detergent in the micellar phase (the total minus the critical micelle concentration) and is expressed on the bulk aqueous scale.

Glutaraldehyde cross-linking

Glutaraldehyde cross-linking experiments were carried out with the same protein samples that were used to set up sedimentation equilibrium experiments. Each SN-EpoR TM construct was reacted in 11 mM C14SB detergent at a concentration of 43 μM protein with 0.1% (v/v) glutaraldehyde for the time indicated, followed by neutralization by the addition of SDS sample buffer. Samples were assayed on a 20% polyacrylamide Phast gel and stained with Coomassie brilliant blue R-250.

Acknowledgements

This work was supported by a grant from the NSF (MCB0423807) and by a Career award from the Department of Defense (DAMD17-02-1-0427).

References


*Edited by J. E. Ladbury*