

Thrombopoietin receptor activation: transmembrane helix dimerization, rotation, and allosteric modulation

Erin E. Matthews,^{*,1,2} Damien Thévenin,^{*,1} Julia M. Rogers,^{*} Lisa Gotow,^{†,2} Paul D. Lira,^{†,2} Lawrence A. Reiter,[†] William H. Brissette,[†] and Donald M. Engelman^{*,3}

^{*}Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut, USA; and [†]Pfizer Global Research and Development, Groton Laboratories, Groton, Connecticut, USA

ABSTRACT We report how rotational variations in transmembrane (TM) helix interactions participate in the activity states of the thrombopoietin receptor (TpoR), a type 1 cytokine receptor that controls the production of blood platelets. We also explore the mechanism of small-molecule agonists that do not mimic the natural ligand. We show, by a combination of cysteine cross-linking, alanine-scanning mutagenesis, and computational simulations, that the TpoR TM dimerizes strongly and can adopt 3 different stable, rotationally related conformations, which may correspond to specific states of the full-length receptor (active, inactive, and partially active). Thus, our data suggest that signaling and inactive states of the receptor are related by receptor subunit rotations, rather than a simple monomer-dimer transition. Moreover, results from experiments with and without agonists *in vitro* and in cells allow us to propose a novel allosteric mechanism of action for a class of small molecules, in which they activate TpoR by binding to the TM region and by exploiting the rotational states of the dimeric receptor. Overall, our results support the emerging view of the participation of mutual rotations of the TM domains in cytokine receptor activation.—Matthews, E. E., Thévenin, D., Rogers, J. M., Gotow, L., Lira, P. D., Reiter, L. A., Brissette, W. H., Engelman, D. M. Thrombopoietin receptor activation: transmembrane helix dimerization, rotation, and allosteric modulation. *FASEB J.* 25, 000–000 (2011). www.fasebj.org

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THE THROMBOPOIETIN RECEPTOR (TpoR) regulates the proliferation of multipotent hematopoietic bone marrow stem cells, their differentiation into mature megakaryocytes, and the resulting production of platelets in response to its ligand, thrombopoietin (Tpo) (1–3). On ligand binding at the extracellular domain, TpoR triggers the activation of an associated cytoplasmic kinase, JAK2. Subsequent phosphorylation of tyrosine residues in the cytoplasmic domain of TpoR provides docking

sites for signaling proteins such as the transcription factors STAT3 and STAT5 (4, 5).

Like other cytokine receptors, such as the erythropoietin receptor (EpoR), growth hormone receptor (GHR), or interleukins, TpoR consists of an extracellular cytokine binding domain, a single helical transmembrane (TM) domain, and a cytoplasmic domain. However, neither the structure of TpoR nor any of its domains is known; consequently, models for its activation are based on the related EpoR and GHR (6). Traditionally, it has been thought that binding of a single ligand to 2 receptor monomers initiates dimerization and is sufficient for activation, but recent studies have shown that mEpoR, GHR, and the prolactin receptor (PrLR) can form homodimers on the cell surface independently of ligand binding (6–10). However, the predimerization of the receptor is insufficient to trigger signaling without ligand. Therefore, a new model for the activation of cytokine receptors has emerged in which the ligand binds to an inactive but preformed receptor homodimer. Ligand binding induces conformational changes that reorient the receptor molecules, allowing the cytoplasmic domains and associated JAK2 molecules to productively phosphorylate each other (6, 11, 12).

The TM domain is thought to be involved in the process of receptor dimerization and activation of cytokine receptors. It has been shown that the isolated TM domain of the EpoR has a strong potential to

¹ These authors contributed equally to this work.

² Current address: E.E.M., Lead Discovery, Bristol-Myers Squibb Co., Research Pkwy., Wallingford, CT 06492, USA; L.G., Department of Microbiology, University of Colorado Denver, 12800 E. 19th Ave., Aurora, CO 80045, USA; P.D.L., Pfizer La Jolla, Department of Oncology, 10777 Science Center Dr., San Diego, CA 92121, USA.

³ Correspondence: Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 208114, New Haven, CT 06520-8114, USA. E-mail: donald.engelman@yale.edu

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self-interact (13) and that for the full-length EpoR expressed at the cell surface, the TM domains can play a role in receptor dimerization in the absence of ligand (7). Furthermore, constraining their TM domains in specific orientations can activate the EpoR or the GHR (6, 14), and it is therefore hypothesized that activation involves a relative rotation of the TM helices. Thus, the dimerized TM domains are believed to adopt different conformations in the inactive, unliganded receptor and in the active, ligand-bound receptor.

Immune thrombocytopenia (lack of sufficient platelets in the blood) can be caused by decreased platelet production or an increased rate of platelet removal or destruction, and it frequently arises during chemotherapy or in certain diseases, including chronic liver disease and AIDS (15). Recently, small nonpeptidyl molecules that activate the human TpoR but do not mimic the native ligand have been identified (15–22). Notably, the FDA recently approved eltrombopag (Promacta/Revolade; GlaxoSmithKline, London, UK) as the first small-molecule TpoR agonist for the treatment of idiopathic immune thrombocytopenia (23, 24). Even though it has been discovered that a histidine residue (His499) in the TM domain of hTpoR is critical for the binding and activity of these small-molecule agonists (15, 19, 25, 26), their mechanism of action is not yet well understood.

In addition, a mutation at Ser505 in the TM domain to an asparagine residue results in constitutively active receptors (27). It leads to familial essential thrombocytopenia (FET), a hereditary disease characterized by excessive production of platelets (28). Even though it is believed that Asn505 results in strong receptor dimerization (27, 29), its role is not fully understood.

Our study is motivated by the desire to understand the activation of TpoR and to define the mechanism of TpoR small-molecule agonists. To do so, we consider several unanswered questions: Does the TpoR TM domain promote homodimerization of the receptor in the absence of ligand? Which TM residues are involved in stabilizing receptor interactions? Does this interface change with activation by mutation or drug binding? To address the first two questions, we study the TM interaction, model the interface, and use cross-linking to identify key contacts. To address the last question, we investigate the role of S505N in FET and the mechanism of action of a piperidine-4-carboxylic acid TpoR small molecule agonist from Pfizer (compound PF; Pfizer, Ann Arbor, MI, USA; refs. 20, 30, 31).

We show that the TM domain of the human TpoR dimerizes strongly and that the TM domains of TpoR can adopt 3 different stable, rotationally related conformations involving distinct sets of TM residues. Similar to other cytokine receptors, our data suggest that signaling and inactive states of the receptor are related by receptor rotation rather than a simple monomer-dimer transition. Interestingly, our data also suggest a mechanism of action for a TM-acting TpoR agonist. Overall, these results give new insight into the activation mechanism of TpoR and support the emerging

importance of the TM domain in cytokine receptor activation and as a site of modulation by small molecules in single-pass TM receptors.

MATERIALS AND METHODS

Cloning and mutagenesis

Cloning of the TM of TpoR for bacterial expression

An oligonucleotide encoding the predicted TM domain of the human TpoR (⁴⁹¹WISLVTALHLVGLSAVLGLLLLRW⁵¹⁵) was synthesized (W. M. Keck Facility; Yale University, New Haven, CT, USA), amplified by PCR to include *NheI* and *BamHI* restriction sites, and cloned into the bacterial expression vector pccKAN (32), resulting in a TOXCAT fusion protein. The plasmid was transformed into NT326 (*malE*⁻) cells (a MBP-deficient *Escherichia coli* strain). This expressed construct consists of an N-terminal DNA-binding domain of ToxR, a TM domain, and the periplasmic maltose-binding protein (MBP).

Cloning of full-length hTpoR for mammalian expression

The pMIG (33) retroviral vector, provided by Luk VanParjis (Massachusetts Institute of Technology, Cambridge, MA, USA), was modified by removing the IRES and EGFP domains and replacing the original MCS with one containing unique *BglII*, *EcoRI*, *HindIII*, *BamHI*, and *Clal* sites. Human TpoR (NM_005373.1, GI:4885490) was PCR cloned using primers incorporating a 5' *EcoRI* and 3' *Clal* sites. The TpoR PCR fragment and MCS/pMIG(-IRESE GFP) vector were each digested with *EcoRI* and *Clal* and ligated.

Site-directed mutagenesis was carried out using appropriate primers and the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the accompanying protocol. All clones were verified by DNA sequencing.

Chloramphenicol acetyl transferase (CAT) activity measurements

Constructs transformed into NT326 were cultured in LB medium with ampicillin until OD₆₀₀ reached 0.6. Cell-free extracts were prepared by pelleting 200 μ l of cells at OD₆₀₀ 0.6, resuspending in 500 μ l of 0.1 M Tris (pH 8.0), and lysing with 20 μ l of 100 mM EDTA, 100 mM dithiothreitol (DTT), 50 mM Tris (pH 8.0), and 5 μ l toluene at 30°C for 30 min. The cell-free extract was then diluted 80-fold before being used in the CAT assays, and incubated at 37°C with tritiated acetylated chloramphenicol (CAM) and *n*-butyryl coenzyme A. After 90 min, the reaction was stopped by partitioning the [³H]CAM-butryl CoA complex into xylene. The organic phase was washed and quantified using the radiolabel. All measurements were performed \geq 4 times.

malE complementation assay

ToxR-TM-MBP chimera-expressing NT326 cells were cultured on M9 agar plates containing 0.4% maltose, 1% ion agar, and Amp. Only constructs with the correct orientation (MBP in the periplasm) grow on the minimal medium with maltose. All TOXCAT constructs showed a correct membrane insertion (data not shown). It is important to note that the TOXCAT construct places the TM domain of TpoR in opposite topography in the membrane (C terminus out) than

would be found in its native cell membrane in platelets (C terminus in).

Cysteine cross-linking with TOXCAT constructs

Disulfide cross-linking was carried out on whole *E. coli* cells using Cu(II)-(*o*-phenanthroline)₃, an oxidizing agent that serves as a disulfide bond catalyst. The Cu(II)-(*o*-phenanthroline)₃ stock solution was prepared fresh before each use by mixing 180 μ l of a 1 M DMSO solution of 1,10-phenanthroline (Sigma-Aldrich, St. Louis, MO, USA) with 60 μ l of a 1 M aqueous solution of Cu(II)SO₄ (Sigma-Aldrich), followed by dilution with 760 μ l of water. This stock solution was then diluted in PBS to obtain the working solution. Constructs transformed into NT326 were cultured in LB medium with ampicillin until OD₆₀₀ 0.6. Cells were harvested by pelleting 200 μ l of culture for 3 min at room temperature and 7000 *g* and were resuspended in 50 μ l of 75 μ M of cross-linker, Cu(II)-(*o*-phenanthroline)₃ in PBS. The cross-linking reaction was performed at room temperature for 15 min. Concentration-dependence and time-course experiments were performed to determine the optimum concentration of oxidizing agent and incubation time. The reaction was quenched with 10 mM ethylenediamine tetraacetic acid (EDTA) and 25 μ l of LDS (4 \times) NuPAGE loading buffer (Invitrogen, Carlsbad, CA, USA). The samples were loaded and run along the SeeBlue Plus2 prestained standard marker (Invitrogen) on a NuPAGE Novex 4–12% Bis-Tris gel using the NuPAGE MES running buffer (Invitrogen). The gel was transferred to nitrocellulose membrane (Hybond-ECL; GE Healthcare, Piscataway, NJ, USA) overnight at 20 V (4°C) and then blotted using 1:500 mouse anti-MBP primary (Invitrogen) and 1:6000 alkaline phosphatase-conjugated goat anti-mouse (Promega, Madison, WI, USA) antibodies. Visualization was achieved using the BCIP/NBT substrate (Invitrogen), which leads to an insoluble black-purple precipitate when reacted with alkaline phosphatase. Images of the gels were analyzed using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA; ref. 34).

Computational searches

The CNS Helix Interactions (CHI) simulation protocol was developed by Adams *et al.* (35, 36) to model TM helix-helix interactions. Possible conformations of TpoR helix dimers were searched by rotating each helix about its long axis (ϕ_1 and ϕ_2 angles) from 0 to 360° in 45° increments (refined to 10° increments in later simulations). Four rounds of molecular dynamics (MD) using torsion angle calculation refinement were performed at each rotational position. Initial crossing angles ($\pm 25^\circ$) were set for the helices to search both right-handed and left-handed interactions. Distance restraints were applied to fix the polypeptide sequence into a helix, and half-parabolic restraints were used to prevent the helices from separating by more than a fixed amount. The interhelical distance was initially set at 10 Å for the calculations (and varied in later simulations to check the robustness of the results). Since the membrane bilayer is a low-dielectric environment, calculations were carried out *in vacuo* without explicit consideration of the surrounding lipid bilayer. The structures obtained after MD simulations were grouped into clusters if ≥ 10 structures had backbone RMSD values ≤ 1 Å. The average structure for each cluster was then calculated and subjected to energy minimization to arrive at the final models.

Mammalian cell expression of hTpoR

hTpoR mutant plasmids were transfected into 293T/17 cells (CRL-11268; American Type Culture Collection, Manassas,

VA, USA) grown to 50–60% confluency in DMEM high-glucose medium (Invitrogen), with 10% heat-inactivated FBS, 1 \times nonessential amino acids, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a T75 flask for retrovirus production. Fugene6 (Roche Applied Science, Indianapolis, IN, USA) was used according to manufacturer's recommendations to transfect 4 μ g of the appropriate MCS/pMIG(-IRESEGFP)_hTpoR mutant plasmid and 4 μ g pCL_10A1 packaging vector (Imgenex, San Diego, CA, USA). The next day, medium was taken off, and 15 ml of warmed UltraCulture (Cambrex, East Rutherford, NJ, USA), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin was added and allowed to incubate overnight. Medium with retrovirus was collected, passed through a 0.22- μ m Steriflip unit (Millipore, Billerica, MA, USA), and concentrated to ~ 200 μ l volume by centrifuging for 15 min at 3000 *g* in Amicon Ultra-15 10⁵ MWCO filter device (Millipore).

IL-3-dependent Baf3/Stat5 murine B-cells with a STAT5 response element regulated β -lactamase reporter, at 2×10^5 /well in a 6-well plate, were transduced by adding 100 μ l of concentrated retrovirus to 2 ml of RPMI 1640 medium, 10% heat-inactivated FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 ng/ml recombinant mouse IL-3 (R&D Systems, Minneapolis, MN, USA), and 8 μ g/ml polybrene. The plate was centrifuged at room temperature for 1 h at 300 *g* and then placed in the incubator overnight. Transduction was repeated the second day using the remaining retrovirus stock (kept at 4°C) and addition of 1 ml medium/polybrene to plate well. On the next day, cells were moved into a T25 flask with 7 ml of RPMI 1640 medium, 10% heat-inactivated FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 ng/ml human TPO (Pepro-Tech, Inc., Rocky Hill, NJ, USA). Cells were expanded and selected in medium with Tpo for 1–2 wk prior to use.

Baf3-TpoR reporter assay

Compound PF and rTpo dilutions were prepared in triplicate in assay medium and delivered to a 96-well black, clear bottom plate (Costar; Corning Life Sciences, Lowell, MA, USA) using the BioMek 2000 (Beckman-Coulter, Fullerton, CA, USA). Next, 20 μ l cells (10^4 cells/well) was added using a Multidrop (ThermoElectron Corp, Waltham, MA, USA), and the plates were incubated for 5 h at 37°C, 5% CO₂. Controls included wells with cells plus 150 ng/ml hTpo in assay medium, cells plus assay medium, and assay medium only. LiveBLAzer-FRET B/G (CCF4-AM; 10 μ l; Invitrogen) was added to each well, and the plates were incubated in the dark, at room temperature for 1 h. The plates were read on an LJL Analyst (Molecular Devices Corp., Sunnyvale, CA, USA) equipped with the 405-20 excitation filter, 2 emission filters (blue channel 460-40 and green channel 530-10) and a 425 dichroic filter. A stimulation index (SI) was calculated for both hTpo and compound PF using the formula (460:530 ratio for drug sample)/(460:530 ratio for no drug or hTpo control sample). EC₅₀ was calculated by plotting SI drug:hTpo control ratio.

RESULTS

TM domain of TpoR self-associates in cell membranes

The TM domain of the TpoR is thought to play a significant role in receptor dimerization. To explore the chemistry of TM homodimerization, we employed the TOXCAT assay (32), which measures the associa-

tion strength between TM helices in the *E. coli* inner membrane environment. The resulting data are shown in **Figure 1B**. For comparison, the signals obtained with the strongly dimerizing TM sequence of glycoporphin A (GpA-WT) and its destabilizing mutant G83I (GpA-G83I) are shown. The TpoR TM sequence shows a strong tendency to form homodimers in a native biological membrane.

Cysteine cross-linking reveals that TpoR TM forms a left-handed dimer

To identify specific residues that mediate the self-association of the TpoR TM domain, we used cysteine-scanning mutagenesis (from Ile492 to Leu513) of the TOXCAT expressed TM sequence and carried out disulfide cross-linking on whole *E. coli* cells using Cu(II)-(*o*-phenanthroline)₃ as a membrane soluble oxidizing agent. The results are shown in **Figure 2A**. Of these mutants, only constructs with cysteine at positions 492–498, 503, 506, 510, 513, and 515 formed disulfide-linked dimers. Significantly, the cross-linking pattern seen with Gly503, Ala506, Leu510, and Leu513 is consistent with a parallel left-handed helical interface with contacts every third or every fourth residue (Fig. 2B). The disulfide-bonded dimers observed for S492-L498 and W515 are continuous rather than periodic, suggesting that these residues are less conformationally constrained, perhaps as a result of being located outside of the hydrophobic core of the membrane (Fig. 2A).

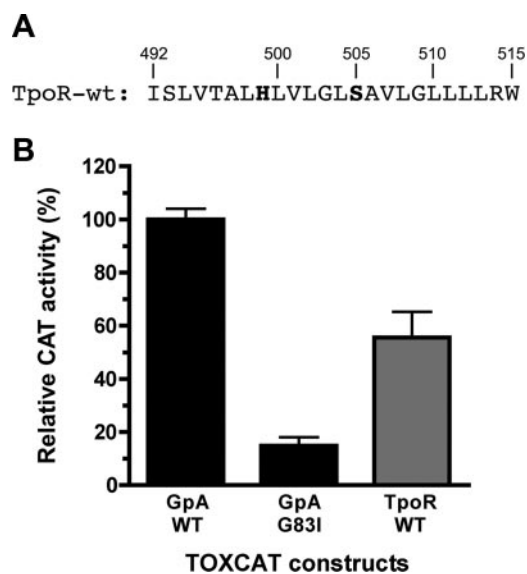


Figure 1. TM domain of TpoR forms relatively strong homodimers. *A*) Amino acid sequences corresponding to the TM domain of the wild-type Tpo receptor (TpoR-wt). *B*) Quantitative TOXCAT assay of TM dimerization: relative CAT activities for the TOXCAT constructs corresponding to the TM sequences of TpoR-wt. Level of CAT activity is directly correlated to the strength of association. Strong dimer formed by glycoporphin A (GpA-wt) and its disruptive mutant (GpA-G83I) are shown for comparison. Results are normalized to GpA-wt. Error bars = sd; ≥ 4 measurements.

In addition, H499C does not form cross-linked dimers, despite being predicted to be in the dimer interface (Fig. 2B), which agrees with the fact that replacing His499 by an alanine or a cysteine residue does not affect the association strength of the TpoR TM domain (Fig. 2C). It indicates that His499 is not critical for oligomerization of the TM domain.

When sequences corresponding to single cysteine mutants at positions 503–513 are assayed for their dimerization potential with the TOXCAT assay, we find that all constructs display a signal comparable to that of the wild-type except for G503C, A506C, L510C, and L513C, which form slightly stronger dimers (Fig. 3A). The fact that these dimers are only slightly stronger than the wild-type sequence dimers may be due to improved packing interactions along the interface, as they are too weak to result from the strong covalent bond of a disulfide-linked dimer. We also note that these 4 constructs are the only ones to show faint dimeric bands without cross-linking treatment (Fig. 3B). This result does not indicate that the cysteines drive the association, but rather that the helices are oriented in a specific manner, allowing cysteine residues to be close enough to form disulfide bonds even without an oxidizing agent. Furthermore, the same cross-linking pattern is observed when the single cysteine mutants are ran on gel in presence of iodoacetamide (*i.e.*, a cysteine blocking reagent; Fig. 3C), establishing that the dimers are formed in the bacterial membrane and not in the SDS buffer, which is a strong promoter of air oxidation of cysteine. Taken together, these results demonstrate that the interhelical disulfide bonds are not simply due to random collisional trapping of cysteine residues but due to specific interactions that map the helix dimer interface.

Naturally occurring S505N-activating mutation causes increased dimerization

To investigate the role of the S505N mutation in FET, a TOXCAT construct containing the mutation was prepared. **Figure 4A** demonstrates that mutating Ser505 to an asparagine significantly strengthens the association of the TM domain of TpoR compared to the wild-type sequence. This increase in association strength is most likely to arise from the formation of an interhelical hydrogen bond between the two asparagine residues, since mutating S505 to other polar residues, such as Asp, Glu, and Gln, increases the association strength as well (Fig. 4A). It is also known that asparagine can mediate strong TM domain interactions and promote the formation of dimers (37). However, S505 is not located in the interface identified by cysteine cross-linking (Fig. 2A, B), implying that N505 may reorient the contact region between the two helices.

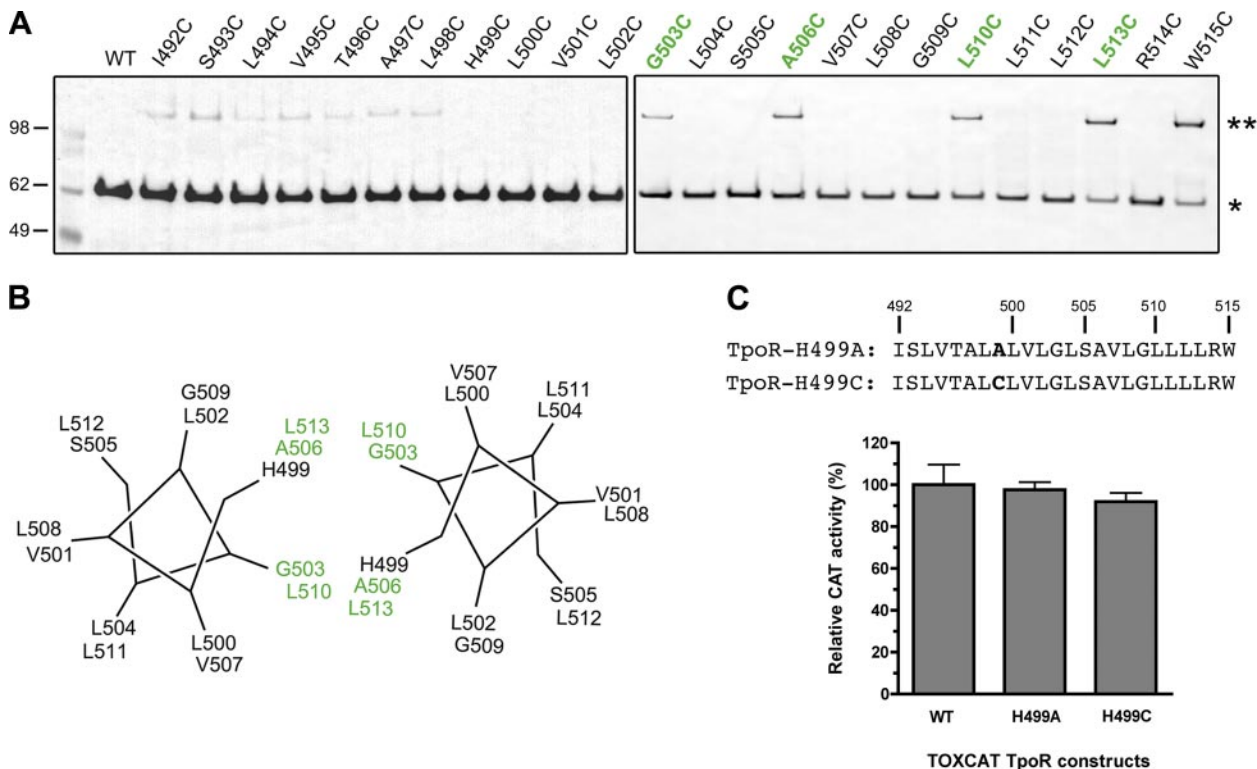


Figure 2. TM domain of TpoR associates as a left-handed dimer with a specific contact interface. *A*) Representative example of disulfide cross-linking of the TM domains of TpoR in whole *E. coli* cells using Cu(II)-(*o*-phenanthroline)₃ as a membrane soluble oxidizing agent (75 μ M for 15 min at room temperature). Constructs were immunoblotted following SDS-PAGE using an anti-MBP antibody. Blot reveals bands corresponding to the expected monomeric (*) and dimeric (**) forms. *B*) Helical wheel representation of the left-handed homodimer formed by 2 wild-type TpoR TM domains. Positions identified by cross-linking as being interfacial (*A*) are highlighted in green. *C*) Relative CAT activities for TOXCAT constructs corresponding to the TM sequences of TpoR-H44A and TpoR-H499C. Results are normalized to TpoR-wt. Error bars = sd; ≥ 4 measurements.

S505N promotes the formation of a different dimerization interface

To determine whether S505N is part of an alternative helix dimer interface, we performed cysteine-scanning mutagenesis of the TM sequence corresponding to TpoR-S505N and carried out disulfide cross-linking as described above. The results are presented in Fig. 4B. Interestingly, mutants with a cysteine at positions 502, 509, or 512 now form disulfide-linked dimers, whereas a dimer band is no longer observed at position 503. The cross-linking pattern displayed at positions 502, 509, and 512 is consistent with a left-handed helical interface with N505 at the contact region (Fig. 4C). Moreover, the proportion of dimers is significantly higher than the one observed without the S505N mutation (Fig. 2A). It is also important to point out that the presence of cysteine residues does not further increase self-association strength, as shown by the TOXCAT assay (Supplemental Fig. S1A). In addition, dimer bands are observed at some of the positions previously noted, namely at 506, 510, and 513, possibly from equilibrium between the two identified helix-dimer conformations. Dimer bands can be seen at positions 492–498 and 515 (Fig. 4B); as previously noted, we take this as evidence that this region may lie outside the hydrophobic core and so is less conforma-

tionally constrained. These results support the idea that N505 can mediate a strong interaction between the two helices, albeit with a different helical interface than noted in our first cross-linking experiments.

Computational searches of the TM dimeric interfaces

To enrich the interpretation of the data, we have conducted a computational search for all chemically plausible symmetric and nonsymmetric dimer structures for the helical TpoR TM domain. The sequence of the hTpoR TM domain from Ile492 to Leu513 was studied with MD simulations using the program CHI. The MD simulations were not constrained by any data from other experiments. In the initial set of simulations, the two helices were separated by 9–10 Å, sampled at 45° increments, and refined with torsion angle calculations. CHI predicts 10 chemically reasonable or possible dimer structures, 5 of which have left-handed crossing angles (Fig. 5A). Only 2 of those left-handed structures have 2-fold symmetrical interfaces (Fig. 5B, C). Of those two symmetrical structures, one (cluster 1) closely resembles the interface detected by cysteine cross-linking in the wild-type hTpoR TM construct by positioning His499, Leu502, Ala506, Leu510, and Leu513 at the dimer interface. The other (cluster 2) corresponds to a structure that is rotated by

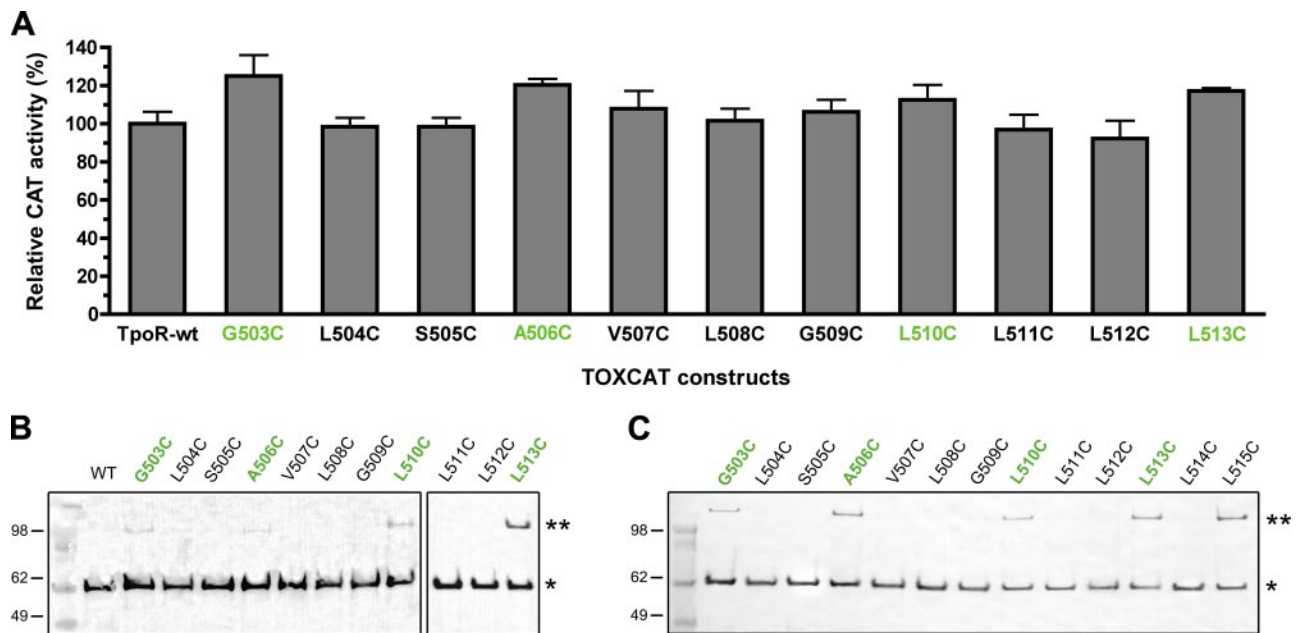


Figure 3. Interhelical disulfide bonds are due to specific interactions that map the helix dimer interface. *A*) Comparison of the dimerization potential of the wild-type and cysteine mutant TpoR TM domains. Results are normalized to TpoR-wt. Error bars = SD; ≥ 4 measurements. *B*) Immunoblotting of the wild-type and cysteine mutant TpoR TOXCAT constructs expressed in *E. coli* without cross-linking treatment. Positions identified by cross-linking as being interfacial (Fig. 2A, B) are highlighted in green. *C*) Immunoblotting of cysteine mutant TpoR TOXCAT constructs expressed in *E. coli* after cross-linking and 50 mM iodoacetamide treatments.

150° and positions His499 outside the contact interface. Consequently, cluster 1 and cluster 2 are henceforth referred to as the “histidine-in” and “histidine-out” conformations, respectively.

A small-molecule TpoR agonist requires specific TM residues for its activity

One of our goals is to understand how small-molecule compounds like the drug eltromopobag, thought to bind the TM domain of TpoR, can lead to receptor activation. A new class of these molecules has been identified (38), and we used one of these, a piperidine-4-carboxylic acid (compound PF in Fig. 6A) to probe TpoR TM domain interactions and the mechanisms involved in receptor activation. To achieve this aim, we performed alanine-scanning mutagenesis of the residues in the TM region of the full-length receptor expressed in IL-3 dependent Baf3/Stat5 murine B-cells. Each alanine mutant receptor was tested for its response both to the recombinant hTpo and compound PF, using a STAT5 response element-regulated β -lactamase reporter assay (Tables S1 and S2). Every position from I492 to L513, except for 497 and 506 (which are already alanine) and 499, were mutated to alanine. H499A was not tested because it is known to be essential for the activity of this family of compounds (19, 31). The results presented in Fig. 6B (blue bars) and Table S1 show that the alanine mutant receptors respond to hTpo as well as the wild-type receptor does. The slightly lower signals observed with V501A, L502A, and L513A are due to a smaller number of cells present in the well during the activity assay, because each of these mutants

responds to hTpo as efficiently as the wild-type receptor (Supplemental Fig. S2). These data confirm that substituting alanine residues in the TM domain does not disturb the wild-type receptor activity significantly. Figure 6B (red bars) and Supplemental Table S2 also show that compound PF activates wild-type TpoR and many of the alanine mutants. However, 5 of the alanine mutants, namely L494A, V495A, L498A, L504A, and L508A, show almost complete loss of activity in response to compound treatment, despite normal activation by hTpo, suggesting that these positions are important for compound activity. Remarkably, when mapped to the various possible TpoR TM domain structures, the sensitive alanine mutants are all found on one helical interface that corresponds to one side of the histidine-out dimer structure, as shown in Fig. 6C. Interestingly, compound PF does not affect helix-helix association strength in TOXCAT (Supplemental Fig. S1B). This result is important and suggests that small drug compounds may not act as previously thought (*i.e.*, bind to the TM His499 and alter the monomer-dimer equilibrium).

DISCUSSION

We show that the TM domain of the TpoR has a strong tendency to self-associate in the cell membrane (Fig. 1B), as does the TM in the related cytokine receptor, the EpoR (13). We also find that activation involves rotations of the receptor TM dimers, similar to the proposed mechanisms for the GHR and the EpoR. Further, we suggest that small drug-like molecules can

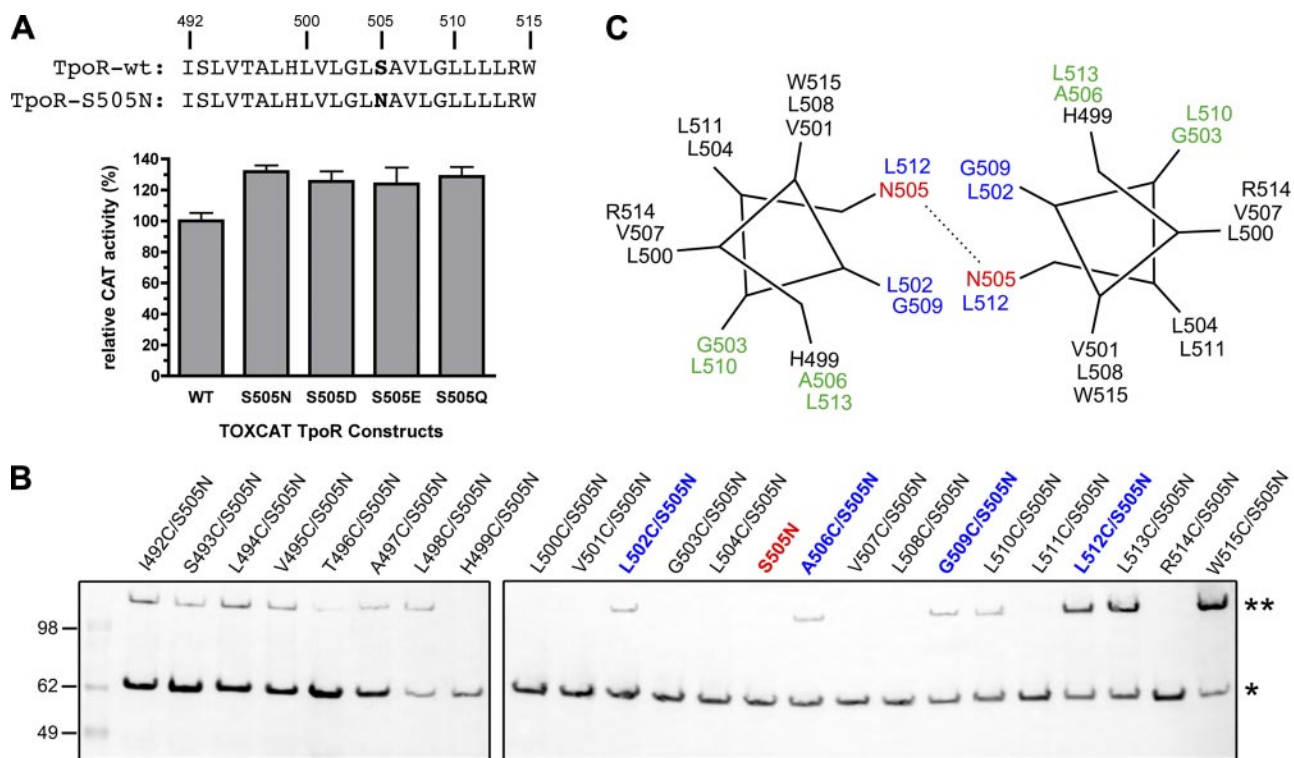


Figure 4. S505N causes increased dimerization and promotes the formation of a different dimerization interface. *A*) Comparison of the relative CAT activities for the TOXCAT constructs corresponding to the TM sequences of TpoR-wt, TpoR-S505N, TpoR-S505D, TpoR-S505E and TpoR-S505Q. Results are normalized to TpoR-wt. Error bars = sd; ≥ 4 measurements. *B*) Representative example of disulfide cross-linking on whole *E. coli* cells with TpoR-S505N cysteine mutant TOXCAT constructs. Asterisks indicate the monomeric (*) and dimeric (**) forms. *C*) Helical wheel representation of the left-handed homodimer formed by 2 TpoR-S505N TM domains. Asn505 is indicated in red; positions identified by cross-linking as being interfacial (*A*) are highlighted in blue. Positions identified as being part of the contact interface between two wild-type TpoR TM domains (Fig. 2A) are highlighted in green. Dotted line indicates the hydrogen bond that likely forms between the two Asn residues and strengthens the association.

activate the receptor by modulating TM domain rotations in an allosteric mechanism that is independent of the native ligand thrombopoietin and its binding site (20, 31).

Using site-directed disulfide cross-linking, we show that the TM domain of TpoR forms parallel left-handed dimers mediated by 4 specific residues: Gly503, Ala506, Leu510, and Leu513 (Fig. 2A). MD simulations confirm the specificity and stability of this dimer interface (Fig. 5). It defines the first stable helical dimer conformation in our study, which positions His499 at the contact interface between the two helices (histidine-in conformation). Our results indicate that His499 is not critical for TM oligomerization, which agrees with the fact that His499 is not required for Tpo-induced TpoR activation (25). The data also reveal that the most N-terminal portion of the predicted TM domain (Ile492 to Leu498) is continuously accessible to cross-linker, which likely indicates that it is in a flexible region located outside of the membrane hydrophobic core. These results agree well with an NMR study reporting that the helical hydrophobic portion of the TM domain of TpoR extends from Ala497 to Leu513, and residues preceding Ala497 are not continuous with the TM helix (25).

Introduction of the constitutively activating mutation S505N in the TpoR TM domain significantly increases

the strength of self-association (Fig. 4A), suggesting that the S505N mutation causes constitutive receptor activation by creating a tightly associated dimer complex. This result is consistent with the FET disease. Indeed, a recent study shows that signal transduction is preserved in a truncated version of the Asn505 mutant receptor lacking the extracellular domain and that autonomous TpoR dimerization is likely due to strong residue polarity in the TM domain (29). However, a key point of our findings is that S505N occurs on a different helical dimer interface than the histidine-in conformation. Our disulfide cross-linking data reveal that the TM domain corresponding to the S505N mutant forms parallel left-handed dimers, mediated by specific residues different from the ones identified with the wild-type TM domain: Leu502, Asn505, Gly509, and Leu512 (Fig. 4B, C). It defines a second TM dimer conformation (Fig. 3C), which differs from the one adopted by the wild-type sequence (Fig. 2B) by a rotation of $\sim 100^\circ$ (1 residue in a left-handed helix interaction). Furthermore, since S505N causes strong association of the TM domains and constitutive activation in the full-length receptor, it is likely that this second dimer interface corresponds to an active form of the receptor.

Alanine-scanning mutagenesis studies (Fig. 6B) reveal that compound PF activates wild-type TpoR nearly

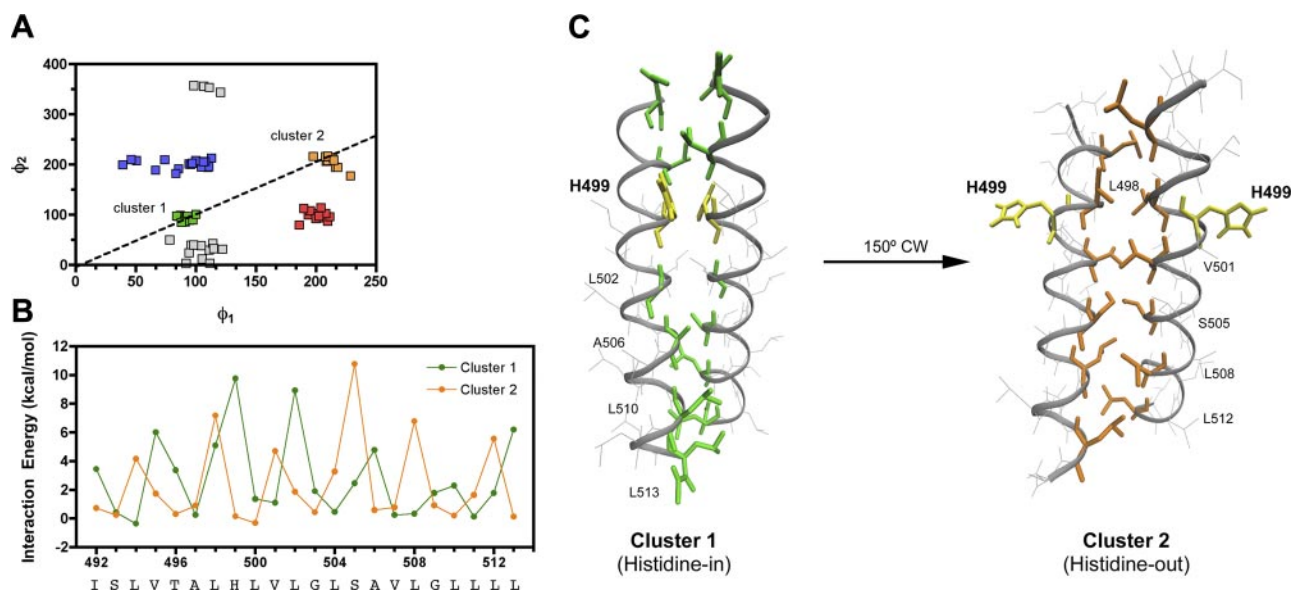


Figure 5. MD simulations: low-energy symmetric TpoR conformations corresponding to the interfaces of contact identified experimentally. *A*) Computational search for low-energy dimer structures of the TpoR TM region from Ile492 to Leu513. Five chemically possible dimer structures with left-handed crossing angles are predicted. Figure plots the final ϕ_1 and ϕ_2 values for those structures that fall into a cluster. Only clusters 1 and 2 have 2-fold symmetrical interfaces. *B*) Average contribution of each residue to the helix dimer stability for the energy-minimized average structures derived from clusters 1 and 2. *C*) Low-energy dimer structures with left-handed crossing angle of the TpoR TM region derived from computational searches. Cluster 1 structure corresponds to the interface (green side chains) identified by cross-linking of the wild-type TM of TpoR (Fig. 2). It places H499 at the contact interface (histidine-in conformation). Cluster 2 is rotationally related to cluster 1 by a rotation of $\sim 150^\circ$. It positions H499 (in yellow) outside the interface of contact (histidine-out conformation).

as efficiently as hTpo and that its activity relies on specific positions in the TM domain. While others have shown that the TM His499 is critical for the agonist activity of this class of small molecules, we have further demonstrated that additional TM positions affect efficacy (L494, V495, L498, L504, and L508). These 5 positions have also been shown to affect the activity of a similar small molecule Tpo agonist (SB39472225; ref. 25). Interestingly, these residues are all found on one face of the TM domain and map well the contact interface corresponding to one of the symmetrical conformation identified by computation search (Fig. 5C; cluster 2–histidine-out conformation) and none of them overlap with the first histidine-in helical interface defined in TOXCAT. These sensitive residues identified in the alanine scan may correspond to binding sites for compound PF. However, the affected positions span several spatially separated residues on the opposite helical face from the critical drug binding His499, and positions both N-terminal and C-terminal of His499 are involved (Fig. 5C). So, it is unlikely that a single planar molecule, binding to His499 with its Lewis basic group at one end, is binding to all the positions affected by alanine substitution (31, 38). Thus, we propose, as an alternative complementary explanation, that alanine substitutions at these 5 sensitive TM positions destabilize the active state of the receptor TM dimer interface. This destabilization of the active conformation is not consequential in a thrombopoietin-driven activation response (Fig. 6B; blue bars), which involves larger contact and stabilization surfaces (hTpo is a 37-kDa

hormone), but does affect the ability of a much smaller TM-acting molecule, such as compound PF, to activate the receptor (Fig. 6B; red bars). These results suggest that this additional histidine-out dimer conformation may correspond to another signaling state of TpoR that is slightly rotated from the S505N active state. It also important to point out that in this conformation, His499, which has been shown to confer specificity to compound PF and other small-molecule TpoR agonists (25, 39), faces the bulk lipid (Figs. 5C and 6C) and is then accessible to compound PF.

Thus, we propose that compound PF could stabilize the active/signaling state of TpoR by binding to the exposed His499. Surprisingly, application of the compound did not affect the association strength of the TM domains (Supplemental Fig. S1B). This result was unexpected, because it was thought that the drug compound would bind to the TM His499 and alter the monomer-dimer equilibrium. The recent report from Richter *et al.* (40), wherein application of A β -lowering compounds in a TOXCAT-like system modulates the dimerization strength of the amyloid precursor protein TM domain, establishes the possibility to measure such changes in our studies. Thus, while the lack of effect is a null result, we would, in fact, not necessarily expect to see a change in dimerization strength, as reported by the TOXCAT assay, in our model of rotationally related states, because the helices are associated in both states.

Because TOXCAT is a simplified model system, our experimental data do not allow us unambiguously to confirm whether the histidine-in conformation corre-

1 cytokine receptors (3, 12, 41–44). It has been shown that constitutive activation of GHR was achieved by either introduction of 4 alanines (nominal 40° rotation) within the lower α -helical TM sequence or by addition of 1 alanine residue (nominal 100°) (6); and that the molecular structures determined by MD simulations for the inactive and active orientations of EpoR differ by a rotation of \sim 100° (14).

In the cell membrane, the inactive and active states likely coexist in an equilibrium that allows the binding of compound PF to His499 (which is exposed in the active histidine-out state), leading to a stabilization of the active state and, consequently, to signaling. Binding of Tpo to the extracellular domain would likely have a similar effect in stabilizing an active conformation. The extracellular and juxtamembrane domains certainly play a role in stabilizing these interactions as well (3, 47, 48).

In summary, our studies define three different association interfaces on the TM domain of TpoR: one interface that contributes to the stability of a preformed but inactive receptor dimer, and two additional, rotationally closely related interfaces giving active states of the receptor. The identification of signaling (*i.e.*, Tpo-bound or As505-mediated) and nonsignaling TM contact interfaces in the full-length receptor expressed at the surface of murine B cells is the subject of ongoing investigations. Notably, we propose a novel mechanism of action for a class of small molecules that are able to activate the human thrombopoietin receptor that exploits the rotational states of the dimeric receptor. To our knowledge, it is the first reported allosteric activation mechanism of a cytokine receptor by a small-molecule modulation of the TM domain. F

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