His499 Regulates Dimerization and Prevents Oncogenic Activation by Asparagine Mutations of the Human Thrombopoietin Receptor*

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Ligand binding to the extracellular domain of the thrombopoietin receptor (TpoR) imparts a specific orientation on the transmembrane (TM) and intracellular domains of the receptors that is required for physiologic activation via receptor dimerization. To map the inactive and active dimeric orientations of the TM helices, we performed asparagine (Asn)-scan-mutation studies in and around the TM domain of the murine and human TpoR. Substitution of Asn at only one position (S505N) activated the human receptor, whereas Asn substitutions at several positions activated the murine receptor. Second site mutational analysis of the TM domains of the murine and human TpoR. Substitution of Asn at only one position (S505N) activated the human TpoR and provides additional protection against activating mutations, such as oncogenic Asn mutations in the TM domain.

The thrombopoietin (Tpo) or Mpl receptor regulates megakaryocyte development and supports hematopoietic stem cell survival (1). The Tpo receptor (TpoR) shares general homology with other members of the cytokine receptor family, including the erythropoietin and granulocyte colony-stimulating factor receptors (2). These receptors contain a large extracellular domain that binds signaling hormones in a 2:1 ratio of receptor to hormone (3–5) along with a single membrane-spanning helix and an intracellular domain that associates non-covalently with a tyrosine kinase.

The minimal functional region of the intracellular domain stretches from the boundary of the transmembrane (TM) domain and includes three regions with limited sequence homology: a conserved switch region, Box 1, and Box 2. The switch region contains a hydrophobic motif whose orientation is required for signaling (6, 7). Box 1 is characterized by a proline-rich motif (PXXPXP) that is crucial for cytokine receptors to bind and activate their cognate Janus kinases (8–10). Box 2 is loosely defined as a motif with acidic and hydrophobic residues and is required for maximal cell growth in response to cytokines.

The TpoR has several unique features. First, the extracellular domain is duplicated, and deletion of the membrane-distal domain causes constitutive activation (11). Second, there are 5 amino acids (RWQFP) inserted directly C-terminal to the TM domain whose deletion or mutation causes ligand-independent receptor activation (12). Finally, clinically relevant mutations have been identified that induce constitutive receptor activity in the extracellular juxtamembrane (JM) region (T487A (13)), the TM domain (S505N (14, 15)), and the intracellular RWQFP motif (W515K/L/A (12, 16–19)). The fact that these are clustered in and around the TM domain reflects the importance of this region in the regulation of receptor activation.

It is generally accepted that the active signaling form of cytokine receptors is a dimer, but whether the inactive state is dimeric or monomeric is unknown. Several lines of evidence...
exist to suggest that the erythropoietin receptor (EpoR), one of the most extensively studied cytokine receptors, exists as a preformed dimer, and activation is induced by structural reorganization of the dimer after hormone binding. Crystallographic studies of the EpoR extracellular domain in the presence and absence of ligand suggest that the full-length inactive receptor is dimeric, and hormone binding causes subunit rotation for activation (4, 20). A similar model has been proposed for the structurally related growth hormone receptor (21–23). The first evidence that the TM domain contributes to dimerization of the inactive EpoR came from co-patching measurements using chimeras of the prolactin and erythropoietin receptors in HEK293-derived BOSC cells (24). These studies found the TM domain of the EpoR to be both necessary and sufficient for receptor dimerization in the context of the full-length receptor. This conclusion is supported by biophysical measurements showing that the isolated TM domain of the murine EpoR associates as a dimer (25). However, unlike the TpoR, the TM domain of the EpoR is not a hot spot for activating mutations.

Much less is known about whether the TpoR forms an inactive dimer that changes orientation upon ligand binding. Studies using coiled coil fusions to the TM-cytosolic domain of the murine TpoR showed that one dimeric interface is inactive, one interface mimics the physiologic functions of TpoR, and several murine TpoR showed that one dimeric interface is inactive, one dimer that changes orientation upon ligand binding. Studies using coiled coil fusions to the TM-cytosolic domain of the murine TpoR showed that one dimeric interface is inactive, one interface mimics the physiologic functions of TpoR, and several orientations induce partial levels of activation in vitro and in vivo. However, these studies use an artificial receptor where the large, native extracellular domain dimer (preformed or ligand-bound) is replaced with a short, tightly associated coiled coil that forces symmetric dimerization. Biophysical studies on the association of the human TpoR TM domain using cysteine mutagenesis and the TOXCAT reporter assay indicate that the wild-type TM sequence (residues 491–515) associates as a dimer (27). However, we have recently shown that the 5-amino acid (RWQFP) insert at the intracellular TM-JM boundary of the TpoR impairs dimerization of the upstream TpoR TM helix (28). We further showed that mutation of Trp515 within this motif leads to dimerization of the TM-JM region of TpoR by changing the orientation of the TM helix relative to the membrane bilayer normal (28). Although the studies on isolated TM-JM regions of TpoR reveal that they have a propensity to dimerize, it remains an open question as to whether the full-length receptor is primarily dimeric or monomeric or exists in a monomer-dimer equilibrium.

In this study, asparagine-scanning mutagenesis of the murine TpoR (mTpoR) and human TpoR (hTpoR) was used to modulate the orientation and dimerization of the TM domain of the receptor. The asparagine mutations provide an alternative to the coiled coil dimerization sequences we have previously used to engineer TpoR dimers (26). Asparagine is able to mediate dimerization of TM helices through strong hydrogen bonding interactions and often does so in a sequence-independent fashion (29–31). Moreover, asparagine-scanning mutagenesis addresses the question of whether the clinically observed S505N mutation in humans is unique or just one of many mutations that lead to constitutive activation. We made use of full-length receptors, truncated receptors, and peptides corresponding to only the TM-JM domain for comparative studies between the murine and human receptors. Together, our results provide the structural basis for why the hTpoR cannot be activated by asparagine mutations that readily activate the mTpoR, suggesting an evolutionary mechanism that results in resistance to oncogenic activation of hTpoR.

Experimental Procedures

cDNA Constructs and Cells—Mouse and human TpoR cDNAs cloned in the pMX-IRES-GFP bicistronic vector contained a hemagglutinin (HA) tag at the N terminus. Site-directed mutagenesis was achieved by the overlapping-extension PCR amplification method. The PCR was performed using 1 μl of PfuTurbo DNA polymerase (2.5 units/μl) following the protocol in the QuikChange XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA) as described (28). Sanger sequencing of the entire coding region was used for each construct.

Cell Proliferation Assay—Pro-B interleukin 3 (IL-3)-dependent Ba/F3 cells were maintained in RPMI 1640 medium and 10% fetal calf serum using WEHI cell supernatant as a source of IL-3. TpoR Asn variants subcloned in the pMX-IRES-GFP were used for packaging ectopic retrovirus in BOSC cells. Cells were infected in medium containing Polybrene with packaged retrovirus, and cell pools expressing similar levels (top 15%) of green fluorescent protein (GFP) coded by the bicistronic retroviral vectors were isolated using flow cytometry cell sorting. Cell surface TpoR was assessed by staining with anti-HA antibodies (Covance, Princeton, NJ; 10 μg/ml) and phycoerythrin-conjugated donkey anti-mouse IgG secondary antibodies (28). The levels of TpoR total protein expression were determined by Western blotting using anti-HA antibodies (Roche Applied Science). For determining the maturation state of the N-glycosylated TpoR mutants, we used digestion with endoglycosidase H (EndoH) for 4 h followed by Western blotting with anti-HA antibodies as described (6). Cell proliferation in the presence or absence of cytokines was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI).

Dual-Luciferase Transcriptional Assay of STAT Activation—Transcriptional activation of STAT3 and STAT5 downstream TpoR variants was assessed in γ-2A cells (Janus kinase 2-deficient cells reconstituted with Janus kinase 2) using the Dual-Luciferase assay kit (Promega, Madison, WI). The assay measures luciferase luminescence after transient transfection of the pGRR5 reporter containing the firefly luciferase and STAT3/Luciferase. The luminescence ratio of these two luciferases provides a measure of STAT activation (26, 32). Luminescence was detected in cell lysates with a PerkinElmer Life Sciences Victor-X light analyzer.

Split Luciferase Complementation Assay of Receptor Dimerization—TpoR dimerization was measured using a complementation assay in which two halves of Gaussia princeps luciferase were attached separately to the C terminus of TpoR. The cDNA of TpoR was inserted between the NotI and ClaI restriction sites of the pcDNA3.1/Zeo vector upstream of either the hGluc fragment coding amino acids 1–93 (hGluc1) or the fragment coding amino acids 94–169 (hGluc2) of G. princeps luciferase (33). BOSC cells plated on 24-well plates were co-transfected with the plasmids (coding hGluc1and hGluc2) using

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Lipofectamine (Invitrogen). Twenty-four hours after transfection cells were trypsinized and resuspended in 150 μl of DMEM/F-12 without phenol red (Gibco). Cell samples were flash frozen on dry ice for 10 min and thawed in a water bath at 37 °C for 10 min (cycle was repeated twice). After centrifugation (10,000 × g, 5 min), the supernatants were collected and aliquoted (100 μl/well) in 96-well white plates (Nunc, Thermo Fisher Scientific) to measure the luciferase activity. Native coelenterazine (NanoLight Technology) was reconstituted as a stock solution (1 mg/ml in methanol) and diluted to a final concentration of 20 μM in DMEM/F-12 at room temperature for injection (100 μl). Signal intensities (integrated over 10 s with an injection delay of 2 s) were read on a Victor-X light analyzer. Aliquots of the supernatants were also used to detect the proteins on Western blots (33).

Membrane Reconstitution of TM-JM Peptides for Biophysical Studies—Peptides (40 residues in length) corresponding to the TM domain and JM regions of hTpoR (TRVETATETAWISLVTALHLVLGLSAVLGLLLLRLWQFPAH) and mTpoR (AVRTSGETAWITLVLALLVLSALLGLLLLKLWQFPAH) were synthesized using solid-phase methods. Deuterated (5,5,5-d3) leucine and deuterium-free water were purchased from Cambridge Isotope Laboratories (Andover, MA). Other amino acids and octyl β-glucoside were obtained from Sigma. The crude peptide (5–15 mg) was purified by reverse-phase HPLC on a C4 column using gradient elution. Purified TpoR peptides were reconstituted by detergent dialysis using 1,2-dimyristoyl-sn-glycero-3-phosphocholine and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (10:3) and a ~1:60 peptide-to-lipid ratio. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL) as lyophilized powders and used without further purification. The lipid, detergent, and peptide were dissolved in organic solvent (e.g. hexafluoro-2-propanol or trifluoroethanol). The mixture of lipid, detergent, and peptide in organic solvent was incubated at 37 °C for over 2 h, the solvent was removed by evaporation using a stream of argon gas, and then the sample was placed under vacuum. The dry mixture was rehydrated with phosphate buffer (10 mM phosphate and 50 mM NaCl, pH 7.4) such that the final concentration of octyl β-glucoside was 5–10% (w/v). The rehydrated sample was then stirred slowly for at least 6 h, and the octyl β-glucoside was removed by dialysis using Spectra-Por dialysis tubing (3500 molecular weight cutoff) for 24 h against phosphate buffer at 37 °C. To confirm that the membrane-reconstituted peptides did not aggregate during dialysis, the samples were loaded onto 10–40% (w/v) sucrose gradients and purified. Samples for IR spectroscopy were dialyzed a second time to remove sucrose. The pelletized samples were lyophilized, rehydrated with deuterium-depleted water (50 ± 5% weight), and incubated at 37 °C for 24 h before loading into NMR rotors.

Polarized IR Spectroscopy—Polarized attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectra were obtained on a Bruker IFS 66V/S spectrometer. Reconstituted hTpoR and mTpoR TM-JM peptides (50–100 μg) were layered onto a germanium internal reflection element using a slow flow of air or argon gas directed at an oblique angle to the ATR plate to form an oriented multilamellar lipid-peptide film. IR spectra were obtained using light having either parallel or perpendicular polarization relative to the surface of the germanium plate. The dichroic ratio (RATR) is defined as the ratio between the absorption of parallel (A∥) and perpendicular (A⊥) polarized light. The frequency of the amide I vibration is sensitive to the global secondary structure of the protein, and the dichroic ratio of the amide I band provides information on the orientation of TM helices relative to the plane of the membrane (34). A detailed analysis has been described previously (34). 1000 scans were acquired and averaged for each sample at a resolution of 4 cm⁻¹. The calculation of the helix tilt angles from the observed dichroic ratios was based on a value of the transition moment angle (α) of 39.5° and a maximum membrane order parameter (Smem) of 0.85 (34). The use of the values of α and Smem, is based on measurements of bacteriorhodopsin, an integral membrane protein with seven transmembrane helices of known structure that forms well ordered, two-dimensional membrane arrays. We do not independently know the disorder of the reconstituted TM helices in our samples. Consequently, we assume that the maximum order obtained is equal to that of bacteriorhodopsin obtained from diffraction measurements (35). This results in a lower estimate for the helix tilt angles reported in Table 1 (i.e. greater disorder in our membrane reconstitutions than that found for bacteriorhodopsin would correspond to lower actual tilt angles for an ideal sample with no disorder).

Solid-state NMR Spectroscopy—Deuterium NMR spectra were obtained at a 2H frequency of 55.2 MHz on a Bruker Avance NMR spectrometer using a 4-mm magic angle spinning (MAS) probe. A MAS frequency of 3 kHz was used to increase the number of spinning side bands. Single pulse excitation was utilized using a 4–7.5-μs 90° pulse followed by a 4.5-μs delay before data acquisition. The repetition delay was 0.25 s. A total of 600,000–1,500,000 transients were averaged for each spectrum and processed using a 100-Hz exponential line broadening function. Spectra were obtained at 25 °C.

Two-dimensional dipolar assisted rotational resonance NMR experiments (36) were performed at a 13C frequency of 125 MHz on a Bruker Avance spectrometer. The MAS spinning rate was set in the range of 9–11 KHz ± 5 Hz. The ramped amplitude cross-polarization contact time was 2 ms. Two-pulse phase-modulated decoupling was used during the evolution and acquisition periods. The radiofrequency field strength was 80 kHz. Internuclear 13C... 13C distance constraints were obtained using a mixing time of 600 ms. The sample temperature was maintained at ~50 ± 2 °C. All 13C solid-state MAS NMR spectra were externally referenced to the 13C resonance of neat tetramethylsilane at 0 ppm at room temperature. Using tetramethylsilane as the external reference, we calibrated the carbonyl resonance of solid glycine at 176.46 ppm. The chemical shift difference between 13C of 4,4-dimethyl-4-silapentane-1-sulfonic acid in D2O relative to neat tetramethylsilane is 2.01 ppm.

Results

Asparagine Mutations in the Transmembrane Domain of the Human and Murine TpoR Induce Different Effects—The TM sequences of human and murine receptors are shown in Fig. 1A

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Asparagine substitutions in the transmembrane domain of the human TpoR lead to activation of only one dimeric interface. A, TM sequences of hTpoR (upper) and mTpoR (middle) showing the position of His<sup>505</sup> and the intracellular R/KWQFP motif. A consensus sequence (bottom) of 50 different mammalian TpoRs highlights (red) the positions with sequence identity of <90%. Four of these residues bracket Ser<sup>505</sup> (murine) and Ser<sup>505</sup> (human), which is highly conserved. B, helical wheel diagrams of hTpoR (left) and mTpoR (right) showing the dimer interfaces that would be predicted for interacting helices in a left-handed coiled coil geometry. C, transcriptional activation of hTpoR after substitution of Gly<sup>503</sup>–Leu<sup>510</sup> with asparagine. γ2A cells were transiently transfected with Janus kinase 2 and the wild-type TpoR or the different TpoR mutants, and the activity of these receptors was assessed by measuring luciferase luminescence (in relative luciferase units (rlu)) in the absence or presence of 50 ng/ml Tpo as indicated. Only the S505N mutation triggers ligand-independent dimerization and signaling. D, BaF3 cells stably expressing the hTpoR (Hu TpoR) variants were grown in the absence of cytokine or in the presence of different concentrations of Tpo as indicated. Only TpoR-S505N induced BaF3 autonomous growth. The presented values are averages of triplicates ± S.D. (error bars) for one representative experiment of at least three. *** p < 0.001, one-way analysis of variance.

Along with a consensus sequence for mammalian TpoRs. Across a wide range of species, the TM domain is relatively well conserved. Ser<sup>505</sup> and Trp<sup>515</sup> both have a sequence identity of ~96%. The S505N is associated with familial forms of thrombocytopenia, whereas the W515K/L/A mutations are associated with essential thrombocythemia and primary myelofibrosis. There are only a few residues with less than 90% identity (shown in red). There are four differences between human and mouse in the TM-JM sequence shown. Based on sequence comparison between TpoRs from 50 species, leucine at positions 492 and 500 in the mouse and glycine and arginine at positions 503 and 514, respectively, in the human are the consensus residues.

Asparagine, the residue that leads to constitutive activation upon mutation at Ser<sup>505</sup> (human), is able to mediate dimerization of TM helices as left-handed coiled coils. The early studies of leucine zippers involved Asn residues at the “a” position in the heptad repeat characteristic of a left-handed coiled coil (37, 38). The crystal structure of the GCN4 transcription factor shows that the amide side chain of Asn forms an asymmetric hydrogen bond across the dimer interface (38), and dimerization studies showed that this contributes about −2 kcal/mol of binding energy (39).

Helical wheel diagrams illustrate the possible dimer interfaces in hTpoR and mTpoR (Fig. 1, A and B). Such dimeric interfaces are imposed when the coiled coil domain of Put3 is fused at consecutive positions to the TM domain of mTpoR, thus replacing the extracellular domain (26). There are seven possible symmetric interfaces that might mediate dimerization in the native receptor and that were created by progressive deletion of residues from the N-terminal end of the TM domain after fusion with the Put3 coiled coil (26). These are designated cc-TpoR-0 through cc-TpoR-VI. The cc-TpoR-II interface was found to be inactive, whereas the other interfaces exhibited various degrees of activity.

To investigate whether the full-length TpoR can be activated by asparagine mutations on different TM helix interfaces, TM residues 503–510 were individually substituted with asparagine in the hTpoR, and the receptor activity was monitored using a luciferase assay that reports on STAT5 transcriptional activation (see “Experimental Procedures”). Substitution with Asn only at a single position (S505N) leads to ligand-independent activation of the hTpoR (Fig. 1C). This position is the same as that detected in clinical studies showing only a single site (Ser<sup>505</sup>) where an Asn mutation leads to disease. The results on STAT5 transcriptional activity were confirmed with cell proliferation assays in stable Ba/F3 transduced cells (Fig. 1D). For these measurements, each receptor mutant was retrovirally transduced into Ba/F3 cells and sorted using flow cytometry for similar levels of expression. Only the S505N mutant led to cytokine-independent cell proliferation. Importantly, several substitutions (L508N, G509N, and L510N) led to receptors that
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Asparagine substitutions in the transmembrane domain of the murine TpoR lead to activation of several dimeric interfaces. Leu492→Gly was substituted with asparagine, and these mutant receptors were assessed for their ability to signal in the absence of cytokine. A, y2A cells were transiently transfected with the constructs, and the transcriptional activation of STAT5 was measured by luciferase luminescence (in relative luciferase units). V494N, S498N, L500N, and G502N triggered signaling in the absence of stimulation. B, BaF3 cells stably expressing the different constructs were grown, and cell viability was measured in the absence or presence of the different concentrations of Tpo ligand as indicated. V494N, S498N, L500N, and G502N allow proliferation of BaF3 in the absence of cytokine. The values presented are averages of triplicates ± S.D. (error bars) for one representative experiment of at least three. ***, p < 0.001, one-way analysis of variance between the wild type and each mutant in the absence of cytokine. CTG, CellTiter-Glo.

FIGURE 2. Asparagine substitutions in the transmembrane domain of the murine TpoR lead to activation of several dimeric interfaces. Leu492→Gly were substituted with asparagine, and these mutant receptors were assessed for their ability to signal in the absence of cytokine. A, y2A cells were transiently transfected with the constructs, and the transcriptional activation of STAT5 was measured by luciferase luminescence (in relative luciferase units). V494N, S498N, L500N, and G502N triggered signaling in the absence of stimulation. B, BaF3 cells stably expressing the different constructs were grown, and cell viability was measured in the absence or presence of the different concentrations of Tpo ligand as indicated. V494N, S498N, L500N, and G502N allow proliferation of BaF3 in the absence of cytokine. The values presented are averages of triplicates ± S.D. (error bars) for one representative experiment of at least three. ***, p < 0.001, one-way analysis of variance between the wild type and each mutant in the absence of cytokine. CTG, CellTiter-Glo.

Asparagine-scanning measurements of STAT5 transcriptional activation of the mTpoR reveal a very different picture (Fig. 2). In contrast to hTpoR, asparagine substitutions at positions 494, 498, 500, 501, and 502 in mTpoR stabilize active receptors in the absence of ligand. These results were confirmed by cell proliferation assays in Ba/F3 cells for positions 494, 498, 500, and 502 (Fig. 2B). Asn substitutions at four different positions of the mTpoR clearly lead to activation, whereas S505N (equivalent to S498N in the mouse) is not active in the context of the mTpoR-G502N/L492H double mutant. Indeed, placement of histidine at position 492 in the mTpoR-G502N mutant abolished the activity of the mutant in the absence and the presence of ligand, yielding the same phenotype as the human receptor with the corresponding G509N substitution.

First, mTpoR-G502N (the most strongly activating single Asn mutant) is not active in the context of the mTpoR-G502N/L492H double mutant. Indeed, placement of histidine at position 492 in the mTpoR-G502N mutant abolished the activity of the mutant in the absence and the presence of ligand, yielding the same phenotype as the human receptor with the corresponding G509N substitution.

Second, the mTpoR is not sensitive to eltrombopag, whereas substitution of L492H in the mTpoR imparts sensitivity to eltrombopag as observed previously (40). This confirms that eltrombopag binding requires the presence of histidine in the TM sequence. In contrast, removal of histidine at position 499 in the hTpoR-G509N mutant could restore the activity of this mutant in the presence and absence of ligand. As a control, the human receptor lacking His499 could no longer respond to eltrombopag.

Third, the hTpoR-G509N mutant (equivalent to mTpoR-G502N) does not traffic to the plasma membrane. The hTpoR-G509N mutant is retained intracellularly in an EndoH-sensitive form (Fig. 4), indicating that it contains high mannose N-glycosylation, characteristic of immature receptor retained in the endoplasmic reticulum, or possibly a receptor that cannot progress past the cis-Golgi. In contrast, the double mutation (H499L/G509N) allows the receptor to traffic to the plasma membrane (Fig. 4) and to induce STAT5 activation and autonomous proliferation in Ba/F3 cells (Fig. 3C). H499L appears to also rescue activity for additional Asn mutants in the hTpoR. For example, V507N is partially rescued by the H499L mutation and exhibits weak activity (data not shown). Rescued receptors become EndoH-resistant and are localized at the cell surface (Fig. 4). In the background of the mouse TpoR, the L492H mutation inactivates the Asn mutants and impairs their ability to traffic to the cell surface (data not shown).

Dimerization of hTpoR Causes Straightening of the TM Helix Near His499—The structural basis underlying the mechanism by which His499 influences receptor activation and cell surface expression is a challenge to study in full-length Tpo receptors. However, the original studies describing the influence of Asn mutations on TM helix dimerization were undertaken on isolated TM domains that independently fold into helical secondary structure when reconstituted into membrane bilayers (29–31). Indeed, we have previously shown that the activating S505N and W515K mutations in the context of TM-JM peptides of the human TpoR lead to a change of the tilt angle of the TM helix and induce dimerization, consistent with measure-
ments on the full-length receptor using the split luciferase assay (28). Here, we extend these studies to the G502N and H499L mutants in hTpoR and to the G502N and L492H mutants in mTpoR (Table 1).

Polarized FTIR spectroscopy provides information on the global secondary structure and orientation of membrane-reconstituted TM-JM peptides. Typically, these peptides fold into \( \alpha \)-helical secondary structure that gives rise to an amide I vibrational band at \( \sim 1655 \text{ cm}^{-1} \) in the IR spectrum. The orientation of the TM helix (i.e. the helix tilt angle relative to the bilayer normal; see “Experimental Procedures”) is probed in polarized IR spectra by measurements of the dichroic ratio (\( R_{\text{ATR}} \)). Hydrophobic peptides (20–24 residues in length) typically exhibit dichroic ratios of >3.0, corresponding to a maximum

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**FIGURE 3.** Addition or removal of the TM histidine modifies the activity of the Asn mutants. A, introduction of His instead of Leu at the homologous position in the murine receptor abrogated the activity of the G502N mutant as indicated on the Dual-Luciferase assay performed in transiently transfected \( \gamma \)-2A cells. As a control, eltrombopag (ETPG) was added and was able to activate the L492H receptor. B, substitution of His by leucine restored the activity of the G509N mutant. Ertrombopag was no longer able to activate the receptor lacking His. C, BaF3 cells stably expressing the TpoR variants were assessed for their ability to grow in the absence of cytokine (red) or in the presence of different concentrations of Tpo (gradient of blue: 1, 10, 50, and 100 ng/ml) and eltrombopag (green; 0.7 \( \mu \)g/ml). Cells were all able to growth in the presence of IL-3 (white). The human double mutant G509N/H499L and its murine homolog G502N were able to induce BaF3 autonomous growth in the absence of cytokine. Histograms are averages of triplicates \( \pm \) S.D. (error bars) for one representative experiment of at least three. CTG, CellTiter-Glo; rlu, relative luciferase units.

**FIGURE 4.** TpoR-G509N is not expressed at the cell surface. A, cell surface expression of HA-tagged TpoR was assessed by FACS using an anti-HA.11 antibody. B, BaF3 cells were sorted for the same level of GFP. C, the total level of TpoR protein was assessed by Western blotting with Np40 cell extracts using an anti-HA antibody. D, EndoH and peptide-N-glycosidase F treatment of stable BaF3 cell lines. Sorted BaF3 cells stably expressing the TpoR asparagine variants were treated with EndoH (E) or peptide-N-glycosidase F (P) or not treated (–), and the total level of TpoR protein was assessed using an anti-HA antibody by Western blotting. All the receptors except G509N mutant were resistant to EndoH treatment, revealing that the proteins are mature. The G509N mutant, which was fully degraded by EndoH, is likely immature and does not proceed to the cell surface because of some misfolding that blocks it at the rough endoplasmic reticulum-Golgi transport system. All the receptors depend on N-glycosylation for processing as revealed by the peptide-N-glycosidase F treatment.
helix tilt of ~28°. Lower dichroic ratios indicate something unusual in the TM sequence (or simply reflect poor membrane reconstitutions). Our previous FTIR studies on hTpoR TM-JM peptides revealed dichroic ratios of ~2.8 (28). In Fig. 5, A and B, we show using parallel reconstitutions that mTpoR-WT peptides exhibit a striking increase in the dichroic ratio compared with hTpoR-WT, corresponding to a decrease in the helix tilt angle from 35 to 20°. This change in orientation can be mimicked in the hTpoR by the H499L mutation, which exhibits the same dichroic ratio (3.2) within error as the mTpoR (Table 1).

A second mechanism for increasing the dichroic ratio of the hTpoR-WT TM helix is incorporation of the S505N mutation. The hTpoR-S505N dichroic ratio (3.4) is closer to that of mTpoR-WT (3.3) than to that of hTpoR-WT (2.7). We attribute this increase to a shift in the monomer-dimer equilibrium toward dimer in the S505N mutant (28). The mTpoR-S498N and hTpoR-S505N peptides exhibit similar dichroic ratios (3.3 versus 3.4), suggesting that they adopt similar active orientations.

We also compared the homologous hTpoR-G509N and mTpoR-G502N mutants. The mTpoR-G502N peptide has a much higher dichroic ratio (3.2) than hTpoR-G509N (2.5), consistent with the higher activity of the full-length mTpoR-G502N mutant. The dichroic ratio of hTpoR-G509N is slightly lower than that of hTpoR-WT (2.7). The presence of two polar residues (His499 and Asn509) appears to alter the orientation of the TM helix in the membrane and may be associated with the poor ability of the G509N mutant to traffic. Similarly, introducing a histidine (at the equivalent His499 position) in the murine coiled coil constructs induces TpoR protein instability as observed by loss of activity and protein expression at the cell surface (data not shown).

Previous NMR studies on the structure and orientation of the TM helix of hTpoR found that there was a break in helical secondary structure near Ala497, one turn before His499 (41). The low dichroic ratio of the amide I band observed in FTIR spectra of wild-type hTpoR would be consistent with this observation. To directly probe the helical secondary structure in this region of the peptide in mTpoR and hTpoR, we made use of intramolecular distance measurements using two-dimensional 13C NMR spectroscopy with MAS (36).

Two-dimensional 13C NMR spectra were obtained on the hTpoR and mTpoR TM sequences isotopically labeled with [1-13C]Leu494 or [1-13C]Leu487, [1-13C]Val495 or [1-13C]Val488, and [3-13C]Ala497 or [3-13C]Ala490. These positions just precede His499 and Leu492 in the hTpoR and mTpoR sequences, respectively. In the two-dimensional NMR spectra, intramolecular cross-peaks are observed between 13C sites with a through space separation of ~6 Å. For example, in mTpoR, the 3-13C carbon of Ala490 is in close proximity to the 13C=O groups of Leu487 (4.3 Å) and Val488 (5.4 Å) in α-helical secondary structure, and cross-peaks between these 13C resonances are observed in the two-dimensional NMR spectrum. Fig. 5C shows the full two-dimensional dipolar assisted rotational resonance spectrum of mTpoR-WT where a distinct cross-peak is observed between the [3-13C]Ala490 and [1-13C]Val488. The cross-peak (boxed) between the [3-13C]Ala490 and the 1-13C resonances of Leu487 and/or Val488 is consistent with α-helical secondary structure in this region of the TM-JM peptide. D, row through the [3-13C]Ala490 resonance of mTpoR showing the absence of a cross-peak to either Leu487 or Val488, which is consistent with a kink or break in helical secondary structure in this region of the TM-JM peptide. E, row through the [3-13C]Ala490 resonance of mTpoR showing a strong cross-peak to Leu487 and/or Val488.

### Table 1: Orientation, Secondary Structure, and Association of hTpoR-WT, mTpoR-WT, and Their Mutants

| Sequence       | Dichroic ratio | Helix tilt angle (°) | Leu487-Ala497 structure | Leu494, Val495 13C=O shift | Association |
|----------------|----------------|----------------------|--------------------------|----------------------------|-------------|
| hTpoR-WT       | 2.7 ± 0.1      | 35                   | Extended                 | 172.3, 172.3               | Monomer     |
| S505N          | 3.4 ± 0.1      | 17                   | Helix                    | 175.9, 175.9               | Dimer       |
| G509N          | 2.5 ± 0.2      | 40                   | Extended                 | 172.3, 172.3               | Dimer       |
| H499L          | 3.2 ± 0.1      | 23                   | Helix                    | 176.1, 176.1               | Dimer       |
| hTpoR-WT + eltrombopag | 3.0 ± 0.1      | 28                   | Helix                    | 175.3, 175.3               | Dimer       |
| mTpoR-WT       | 3.3 ± 0.1      | 20                   | Helix                    | 176.3, 176.3               | Dimer       |
| S498N          | 3.3 ± 0.1      | 20                   | Helix                    | 177.2, 177.2               | Dimer       |
| G502N          | 3.2 ± 0.1      | 25                   | Helix                    | 176.6, 176.6               | Dimer       |
| L492H          | 2.6 ± 0.2      | 38                   | Extended                 | 173.1, 173.1               | Monomer     |

* The error in the dichroic ratio corresponds to the standard deviation in three replicate runs.


In the wild-type and mutant TpoR peptides, dimerization can be estimated from deuterium MAS NMR spectra (28). In these experiments, the peptides are deuterated at Leu512 (hTpoR) or Leu505 (mTpoR). Comparison of the deuterium MAS NMR spectra of hTpoR-WT and mTpoR-WT reveals a large difference in the MAS spectra (Fig. 6, A and B). We have previously shown that fast rotational diffusion of the hTpoR monomer results in the loss of the side band pattern and that mutations in the TM and JM regions that lead to dimerization restore the MAS rotational side bands (28). On the basis of the observed side band pattern, we conclude that the monomer-dimer equilibrium in mTpoR-WT is shifted toward dimer compared with hTpoR-WT. The deuterium side band pattern characteristic of dimerization is observed for the activating mutants of hTpoR (S505N and G509N) (Table 1). TM dimerization is also observed for the activating mutants of mTpoR (S498N, G502N, and W508K).

The G. princeps luciferase complementation assay (28, 33) was used to assess the dimerization status of the full-length as well as of truncated mouse and human TpoRs containing TM/intracellular (IC) domains. In this assay, two proteins of interest are fused to complementary fragments of G. princeps luciferase, denoted hGluc1 and hGluc2. If the proteins interact, luciferase fragments are brought together and fold into their native structure, and the reconstituted luciferase enzyme catalyzes the oxidation of the substrate coelenterazine, which can be detected and quantified using luminescence readers.

The C terminus of murine or human TpoR was fused to complementary fragments of G. princeps luciferase (hGluc1 and hGluc2) and transiently co-expressed in BOSC cells where their expression levels were comparable and could be assessed by Western blotting (data not shown). The expression of these constructs individually as expected did not result in reconstitut-
tion of luciferase activity (28). The complementation of hGluc-tagged murine and human full-length TpoR was relatively low and appeared to be at similar levels (Fig. 6C, conditions 1 and 2), indicating that the level of preformed dimers detected by proximity of cytosolic domains is low. Importantly, removal of the extracellular domain resulted in significantly higher dimerization of the mTpoR (condition 4) but not of the hTpoR (condition 3) whose dimerization remained the same compared with the full-length receptor. Thus, the hTpoR TM-IC segment possesses an additional mechanism to prevent its self-dimerization that could be ascribed to His^{499}. Indeed, substitution of His^{499} to leucine allows dimerization of the TM-IC form of hTpoR (condition 5), whereas substitution of Leu^{492} to histidine in the TM-IC form of the mTpoR dramatically decreased dimerization as assayed by luciferase complementation (condition 6).

The results in Fig. 6C for the TM-IC constructs are consistent with the NMR analysis of the TM-JM peptides, namely that His^{499} prevents dimerization of the TM domain of hTpoR in the context of a truncated TpoR lacking the extracellular domain. Using the Dual-Luciferase assay to monitor receptor activity, we show in Fig. 6D that the TM-IC constructs of mTpoR are inactive despite increased dimerization. In the hTpoR constructs, truncation of the extracellular domain abolishes Tpo-dependent activity but still allows activation upon the addition of eltrombopag. This activation is not detected in the H499L mutant.

**Eltrombopag Induces Dimerization of hTpoR TM Helix Peptides**—Comparison of the hTpoR and mTpoR TM-JM peptides shows that the monomer-dimer equilibrium is shifted toward monomer in hTpoR-WT compared with mTpoR-WT (Fig. 6, A and B). Binding of the small molecule agonist eltrombopag induces receptor activation and requires the presence of His^{499} (40). In the absence of activation, His^{499} was previously shown to cause a kink in the TM helix (41). We asked whether this structure changes in the presence of eltrombopag and whether eltrombopag influences dimerization of the hTpoR TM domains. Comparison of hTpoR-WT peptides without (Fig. 6A) and with eltrombopag (Fig. 7B) shows a slight increase in the intensity of the deuterium MAS side bands, consistent with a shift toward the peptide dimer. Furthermore, the secondary structure around His^{499} becomes helical in the hTpoR-WT peptide in the presence of eltrombopag (Table 1). Increased dimerization agrees with the studies on the TM-IC constructs containing the full IC domain that exhibit an increase in activity upon eltrombopag binding (Fig. 6D). We also asked whether substituting His^{499} with leucine, the corresponding residue in mTpoR, would influence dimerization. Indeed, hTpoR-H499L also becomes more dimeric compared with hTpoR-WT (Fig. 7C), consistent with an observed increase in the dichroic ratio from polarized IR measurements (Table 1), whereas the mTpoR-L492H peptide becomes more monomeric compared with mTpoR-WT (Fig. 7A). The dimeric interfaces in Fig. 7, B and C, are predicted to be different between the inactive dimeric and active dimeric states of hTpoR (see below).

**Rotational Orientation of TM Helices in Inactive and Active Dimers**—Interhelical NMR measurements of specifically labeled 41-residue mTpoR peptides (Glu^{481}–Ser^{524}) allow us to probe the interface that mediates dimerization. In these experiments, two mTpoR peptides with complementary ^13C labeling were reconstituted together into 1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dimyristoyl-sn-glycero-3-phosphoglycerol bilayers at a peptide:lipid molar ratio of 1:60. The positions of the labels were selected on the basis of our previous engineered Put3 constructs that defined the inactive and active interfaces.

To probe the inactive dimer interface, two-dimensional ^13C NMR spectra were obtained on mTpoR-WT peptides containing ^13C-labeled Ala^{499} and Gly^{502}. One peptide was labeled with [3-^13C]Ala^{499} and [1-^13C]Gly^{502}, and the other was labeled with [1-^13C]Ala^{499} and [2-^13C]Gly^{502}. Ala^{499} is predicted to be in the inactive interface, whereas Gly^{502} is at the boundary between inactive and active interfaces. We observe a cross-peak between the diagonal 3-^13C and 1-^13C resonances of Ala^{499} (Fig. 8A) and between the diagonal 2-^13C and 1-^13C resonances of Gly^{502} (Fig. 8B). These ^13C-labeled carbons are on different peptides, and the cross-peak intensities are consistent with an interhelical [3-^13C]Ala^{499} to 13C=O distance of ~5.4 Å and an interhelical [2-^13C]Gly^{502} to C=O distance of ~5.2 Å. An intrahelical cross-peak is also observed between the [2-^13C]Gly^{502} resonance and the [1-^13C]Ala^{499} resonance. These ^13C labels are on the same peptide and are estimated to be ~4.4 Å apart in an α-helical geometry. An intrahelical cross-peak between the [3-^13C]Ala^{499} and [1-^13C]Gly^{502} resonances was not observed as the distance between ^13C sites is >6 Å in an α-helix.

To probe the potential active interface(s), parallel two-dimensional NMR experiments were undertaken on mTpoR-S498N (Fig. 8, C and D). In contrast to the results above, we do not observe interhelical Ala^{499}–Gly^{502} or Gly^{502}–Gly^{502} cross-peaks in peptides containing this activating mutation, confirm-
The active orientation of the hTpoR is modeled as a dimer (Fig. 9A, right). In the wild-type full-length receptor, ligand binding drives a shift of the receptor dimer to an active orientation with Ser498 in the interface. Hydrogen bonding interactions involving Asn side chains stabilize this orientation in the S498N mutant (Fig. 9B, right).

The inactive mTpoR also exists in a monomer-dimer equilibrium (Fig. 9B). In the absence of the extracellular domain, the equilibrium shifts toward dimer. The interface mediating the inactive dimer in TM-JM peptides involves packing of Ala499 and Gly502. This interface is the same as that found in the engineered Put3 dimers, both of which contain Ser498 in the interface.

Models of the TM domain of the hTpoR in inactive and active orientations are shown in Fig. 9A. The inactive receptor exists in a monomer-dimer equilibrium. The split luciferase measurements of the full-length receptor along with deuterium NMR measurements of the hTpoR-WT TM-JM peptides indicate that the equilibrium is shifted toward monomer. His499 induces a break in the TM helix. The side chain of His499 is predicted to reside within the headgroup region of phospholipids on the extracellular side of the bilayer. Given the bilayer thickness, this requires the RWQFP motif to partition into the headgroup region of the phospholipids on the extracellular side of the bilayer. The active orientation is modeled as a dimer (Fig. 9A, right). In the wild-type full-length receptor, ligand binding drives a shift of the receptor dimer to an active orientation with Ser505 in the interface. Dimerization is also mediated by hydrogen bonding interactions involving the Asn side chains in the S505N mutant (Fig. 9A, right). In the TM-JM peptides, dimerization induces helical secondary structure in the region just N-terminal to His499 and reduces the overall dichroic ratio (helix tilt angle).

The inactive mTpoR also exists in a monomer-dimer equilibrium (Fig. 9B). In the absence of the extracellular domain, the equilibrium shifts toward dimer. The interface mediating the inactive dimer in TM-JM peptides involves packing of Ala499 and Gly502. This interface is the same as that found in the engineered Put3 constructs (26). In the wild-type full-length receptor, ligand binding also drives a shift of the receptor dimer to an active orientation with Ser498 in the interface. Hydrogen bonding interactions involving Asn side chains stabilize this orientation in the S498N mutant (Fig. 9B, right).
rapid signaling by TpoR. Although our studies reveal clear differences in dimerization in comparisons of (for e.g.) the full-length hTpoR and hTpoR TM-IC-H499L, they open up the question as to how different cell types or membrane environments influence the monomer-dimer equilibrium. For example, there may be differences in the monomer-dimer equilibrium (and hence the level of preformed dimers) between TpoR-expressing cells, namely hematopoietic stem cells and megakaryocyte progenitors, or there may be a shift in equilibrium if the membrane environment changes, such as incorporation of receptors into lipid rafts. The ability of the cell to modulate the monomer-dimer equilibrium would provide an additional control mechanism for receptor activation.

**TM Helix Orientation in TpoR Signaling**—The orientation of the TM and intracellular domains within dimers of single TM receptors is generally important for activation. In both the EpoR (32) and TpoR (26), we have shown using Put3 coiled coil constructs that receptor activity is dependent on the orientation of the TM helix. For mTpoR, only one dimeric interface (cc-TpoR-II) is inactive. The cc-TpoR-II interface is lined with leucines and an AXXG motif and ends with Gln509 within the (K/R)WQFP motif, corresponding to Gln516 in the hTpoR. The AXXG sequence has similarities with the GXXXG sequences known to mediate TM dimerization (43, 44). The close packing of the TM helices due to these small side chains places Gln509 of mTpoR in a position where it can mediate interhelical hydrogen bonding and stabilize dimerization. We ascribe this inactive interface to the same interface that mediates dimerization of the inactive TM-IC constructs of mTpoR. Moreover, we show with NMR (Fig. 8) that the TM-JM peptides (also lacking a large extracellular domain and hence similar to the TM-IC constructs) dimerize via the same interface as in the inactive cc-TpoR-II construct.

In contrast, several interfaces in the Put3 coiled coil constructs have previously been shown to exhibit various levels of activity (26). cc-TpoR-I induced high numbers of platelets without other cytological features of myeloproliferation in bone marrow, and cc-TpoR-V induced a myeloproliferative phenotype with small red blood cells and variable levels of thrombocytosis (26). Thus, the active helix interfaces identified in the Put3 experiments are consistent with the Asn substitutions reported here, at least for the mTpoR. That is, the cc-TpoR-I and cc-TpoR-V interfaces involve the residues where asparagine mutations lead to the strongest activity (V494N, S498N, L501N, and G502N). In cc-TpoR-V, the dimer interface is mediated by an SXXXG sequence, which again allows close helix packing. In our NMR studies, the S498N mutation induces a change in the conformation of the mTpoR peptide dimer, and we lose the contacts between Ala499 and Gly502 characteristic of the inactive dimer (Fig. 8). The low energy structure shown in Fig. 9B corresponds to cc-TpoR-I in which Asn498 forms interhelical hydrogen bonds.

**Eltrombopag Drives Dimerization of hTpoR-WT**—Eltrombopag is a Food and Drug Administration-approved drug used
for the treatment of thrombocytopenia (low platelet numbers). Previous studies have found that eltrombopag can activate hTpoR but not mTpoR (40). Eiltrombopag and related small molecule agonists have been found to interact with His499 (41).

We find that this transmembrane-acting compound increases the dichroic ratio in FTIR measurements, induces helix in the Leu494–Ala497 sequence in the TM domain, and leads to receptor dimerization as assayed by deuterium NMR line shapes. The changes in dimerization induced by eltrombopag (Fig. 7B) are weaker than those observed for the H499L mutant (Fig. 7C). Nevertheless, eltrombopag appears to induce receptor activation in a similar manner as the S505N mutation; namely, both overcome the ability of His499 to inhibit TM dimerization.

**Multiple Levels of Regulation Involving the TM and JM Regions of TpoR**—The wild-type hTpoR has several mechanisms for regulating dimerization of the TM region of the receptor. These involve the extracellular domain, the TM His499 residue, and the intracellular RWQFP insert. Removal of the extracellular domain or the H499L mutation alone does not induce dimerization. Both are required in the same construct. Ligand binding within the extracellular domain or mutations of Trp195 within the RWQFP insert can both overcome these mechanisms, leading to an active receptor conformation. In the mTpoR, removal of the extracellular domain induces dimerization in an inactive conformation. Dual-Luciferase measurements of receptor activation show that the TM-IC constructs of mTpoR are inactive. Thus, dimerization per se does not suffice for activation. Interestingly, the TM-IC constructs differ from the viral Mpl protein of the myeloproliferative leukemia virus by the presence of an extracellular domain of 109 amino acids comprising 43 from the original murine sequence and 66 from the Friend murine leukemia virus. This suggests that the extracellular amino acids in the v-Mpl are able to stabilize the TM-IC portion of the receptor in an active orientation.

Our results reveal that the TM helices in TpoR are not passive elements in ligand-induced dimerization. The TM helices have a stronger propensity to form dimers or higher order oligomers than to remain in a monomeric state. The extracellular, juxtamembrane, and intracellular domains all contribute to modulating TM association. This observation may generally hold for ligand-induced activation of single TM receptors. For example, the TM helices in the ErbB family of receptor tyrosine kinases all have a strong propensity to dimerize (45), and removal of the extracellular domain can lead to active dimers (46, 47) or can trigger intramembrane proteolysis (48, 49). In these receptors, the juxtamembrane sequences play an influential role in dimerization and activation (50). Mutation of Val664 to Gln in the TM sequence of the ErbB2-Neu receptor can induce dimerization and constitutive receptor activation in a manner similar to the S505N mutation in hTpoR (51).

The additional layer of activity control exerted by His499 in hTpoR may reflect the central role that this receptor plays in hematopoiesis. TpoR not only regulates megakaryocyte/platelet development but also contributes to the survival of hematopoietic stem cells, which give rise to all other blood cells. The acquisition of histidine at position 499 during evolution protects the hTpoR from the transforming effect of several Asn TM mutations that are activating in the mTpoR.

**Author Contributions**—S. O. S. and S. N. C. conceived and designed the study, interpreted the data, and wrote the paper. E. L. and J.-P. D. designed and performed experiments (γ2A luciferase assays, Ba/F3 cell retroviral transduction, sorting, cell surface labeling, and proliferation), analyzed the data, prepared the figures, and wrote the paper. J. S. designed and performed experiments (γ2A luciferase assays, Ba/F3 cell retroviral transduction, sorting, cell surface labeling, and proliferation). S. M. M. performed cloning and expression of TpoR constructs. E. L. performed intracellular TpoR metabolism studies. T. S. synthesized and purified the TM peptides. S. D. carried out membrane reconstitutions and FTIR studies. V. G. performed the Gaussia luciferase experiments.

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