NMR Structural Studies of Interactions of a Small, Nonpeptidyl Tpo Mimic with the Thrombopoietin Receptor Extracellular Juxtamembrane and Transmembrane Domains*

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Thrombopoietin (Tpo) is a glycoprotein growth factor that supports hematopoietic stem cell survival and expansion and is the principal regulator of megakaryocyte growth and differentiation. Several small, nonpeptidyl molecules have been identified as selective human Tpo receptor (hTpoR) agonists. To understand how the small molecule Tpo mimic SB394725 interacts and activates hTpoR, we performed receptor domain swap and mutagenesis studies. The results suggest that SB394725 interacts specifically with the extracellular juxtamembrane region (JMR) and the transmembrane (TM) domain of hTpoR. Solution and solid-state NMR structural studies using a peptide containing the JMR-TM sequences showed that this region of hTpoR, unexpectedly, consists of two α-helices separated by a few nonhelical residues. SB394725 interacts specifically with His-499 in the TM domain and a few distinct residues in the JMR-TM region and affects several specific C-terminal TM domain residues. The unique structural information provided by these studies both sheds light on the distinctive mechanism of action of SB394725 and provides valuable insight into the mechanism of ligand-induced cytokine receptor activation.

Thrombopoietin (Tpo)‡ is the primary regulator of megakaryocyte growth and differentiation to platelets (1–3). Binding of Tpo to Tpo receptor (TpoR) triggers activation of the cytoplasmic tyrosine kinases JAK2 and TYK2, which in turn results in the activation of STAT5, phosphatidylinositol 3-kinase, and the mitogen-activated protein kinase signaling pathway. (1–6). Activation of these signaling pathways results in changes in gene regulation, which are thought to promote progression of the TpoR-expression cells along the megakaryocytic pathway.

TpoR is a member of the type I cytokine receptor family, which consists of an extracellular cytokine binding domain, a single transmembrane (TM) domain and a cytoplasmic domain. TpoR is most closely related to erythropoietin receptor (EpoR) and granulocyte colony-stimulating factor receptor (G-CSFR). Considerable evidence suggests that dimerization and conformational change are the essential processes that lead to cytokine/growth factor receptor activation (7–11). Structural studies of EpoR-interacting peptides with a range of activities have revealed that the dimerized EpoR extracellular domain has to be in the correct orientation for downstream signaling events to occur, indicating that receptor dimerization alone is not sufficient to drive receptor activation (12). This is consistent with the observation that EpoR and the growth hormone receptor exist as dimerized but inactive receptors in the absence of ligands (12, 13). EpoR dimerization in the absence of Epo is mediated mainly by its TM domain (7). The isolated TM domain of the EpoR was shown to dimerize, suggesting that the TM domain may be involved in ligand-independent receptor dimerization (7). In addition, the TM domain may also play a key role in mediating receptor activation, as demonstrated by the constitutively active TM domain mutants of the mouse EpoR (mEpoR), human G-CSFR (hG-CSFR), and human TpoR (hTpoR) (14–17).

We investigated the mechanism of action of a small molecule nonpeptidyl Tpo mimic, SB394725. SB394725 is a previously identified hTpoR-specific ligand with an unknown mechanism of action that activates Tpo signaling pathways and promotes differentiation of primary human bone marrow cells to megakaryocytes with an efficacy equivalent to that of recombinant hTpo (3). By performing hTpoR domain swap and mutagenesis studies and using combinatorial approaches of solution and solid-state NMR, we have demonstrated that SB394725 interacts specifically with His-499 in the TM domain and a few distinct residues in the extracellular JMR and the TM domain of the hTpoR.

**EXPERIMENTAL PROCEDURES**

Plasmid Constructs—To generate a STAT response element-containing reporter construct, an oligonucleotide containing eight copies of the STAT response element from the IRF-1 gene...
promoter (5’-TGGTCCTGGGAACTCTGCTACATC-3’) was synthesized and cloned into the pGL2-basic vector. Full-length cDNAs of hTpoR and hG-CSFR were obtained by reverse transcription-PCR from human bone marrow RNA and cloned into the pcDNA3.1/hygro(+) vector. Chimeric receptor constructs were generated by using a PCR-based bridging technique. Briefly, hTpoR and hG-CSFR cDNAs were used as templates to generate G(T)G, G(13 + T)G and T(T)G constructs with a set of primers for each construct. Each primer set contained a forward primer that annealed to the sequence corresponding to the N terminus of the chimeric construct and a reverse primer that annealed to the sequence corresponding to the C terminus of the chimeric construct and a pair of bridging primers that annealed to both the sequences corresponding to the N and C termini of the constructs. The forward and reverse primers for constructs G(T)G and G(13 + T)G were 5’-GTAAGCTTATT-GGCAAGCTGGAAAATGCGACGC-3’ and 5’-GACTCGAGGCTAGTCCCTGGAGACATAGGTCT-3’, respectively. The forward and reverse primers for construct T(T)G were 5’-ACGATATCATGTCACCTCTGCTCCTCATC-3’ and 5’-AGCCCTGAGCTCAAGGTCGGTCACTGGAT-GGAATCTCTTGTGGACCTGCTC-3’, respectively. The bridging primers for constructs G(T)G, G(13 + T)G, and T(T)G were 5’-CCTGACCCCAAGGGGTCGGGAGTT-GGATCTCCTTGGTGACCGCTC-3’, 5’-GACCTCGAGGCTAGTCCCTGGAGACATAGGTC-3’, and 5’-GACTCGAGGCTAGTCCCTGGAGACATAGGTCT-3’, respectively. The resulting PCR products were purified and inserted into the HindIII and Xhol sites for constructs G(T)G and G(13 + T)G and into the EcoRV and Xhol sites for construct T(T)G. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) as described by the manufacturer. A pair of primers containing GCT, GCC, CAC or CAT at the corresponding Leu residue was used to generate the His mutants. Primer pairs containing GCTAGTCCCCCTGGAGCACATAGGTC T-3 and GTAAGCTTAT-GGCAAGCTGGAAAATGCGACGC-3’ were used to generate mutant receptors for alanine-scanning mutagenesis study.

**TGGTCCTGGGAATCTGCCTACATC-3’**

For the selectively15N-labeled peptides, the M9 culture medium with unlabeled (NH4)2SO4 was supplemented with 100–500 mg/liter of the 15N-labeled amino acids and 100 mg/liter of the 15N-labeled Val and Leu (Cambridge Isotope Laboratory). The purity of the peptide was confirmed by SDS-PAGE.

### Sample Preparation

For solution NMR experiments, 15N-labeled hTpoR-(479–519) peptides were dissolved in 400 μl of micelle solution that contained 100 mM deuterated DHPC (1,2-dihexanoyl-sn-glycerol-3-phosphocholine) (Cambridge Isotope Laboratory) and 10% (v/v) H2O at pH 3.6 to a final concentration of 0.5–1.0 mM. Weakly aligned samples were prepared by soaking the solution NMR sample into a dried 5% polyacrylamide gel overnight. The initial gel length of 28 mm was restricted to 20 mm in the Shigemi microcell NMR sample tube (Shigemi Inc., Allison Park, PA). For measurement of the chemical shift perturbations, SB394725 was added directly into 400 μl of the solution NMR sample to a final concentration of 5 mM SB394725, and then the pH of the mixture was readjusted to pH 3.6.

For solid-state NMR experiments, phospholipid bicontinuous cubic samples were prepared by dissolving 15N-labeled hTpoR-(479–519) peptide in the short-chain lipid 1,2-O-dihexyl-sn-glycerol-3-phosphocholine (6-O-PC) and then adding this mixture to the long-chain lipid 1,2-O-ditetradecyl-sn-glycerol-3-phosphocholine (14-O-PC) or 1,2-O-dihexadecyl-sn-glycerol-3-phosphocholine (16-O-PC) (Avanti Polar lipid Inc. Alabaster, AL). The peptide/lipid mixture was vortexed and then left for several days. After the addition of the sample, the mixture was left for several days.

### Structural Studies of Tpo Mimic and TpoR Domain Interactions

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Structural Studies of Tpo Mimic and TpoR Domain Interactions

FIGURE 1. Sequence requirements for SB394725 activity assessed by a luciferase reporter gene assay using different chimeric hTpoRs in HepG2 cells. A, dose-response curves of SB394725-induced activation of different chimeric receptors, T(T)G (red), G(13 + T)G (green), G(T)G (blue), and T(G)G (gray). T and G represent the hTpoR and hG-CSFR sequences, respectively. The G(13 + T)G construct contains 13 extracellular JMR residues in addition to the predicted TM domain of hTpoR. The schematic diagrams of the chimeric receptor are shown with the hTpoR sequences in gray and the hG-CSFR sequences in red. B, sequence alignment of the JMR-TM region of (T)G, (T)H499L, and G(G)G. The key His residue and the corresponding Leu residues are highlighted. C, dose-response curves of SB394725-induced activation of different His mutant chimeric cytokine receptors. Schematic diagrams of the wild-type and His mutant receptors are shown with hTpoR and hG-CSFR sequences in gray and red, respectively. Fold induction was calculated by dividing the luciferase values derived from SB394725 treatment by that of Me2SO treatment.

Dipolar Wave Analysis and Structure Calculation—The experimentally measured residual dipolar couplings (RDCs) were analyzed by MATLAB software to identify helical regions as described elsewhere (22, 23). The RDCs from the overlapped peaks were excluded for the analysis. The 1H,15N RDCs were fitted to sinusoids of a periodicity of 3.6 to characterize the length, curvature, relative orientation, and rotation of α-helices within the hTpoR-(479–519) peptide. Unconstrained nonlinear optimization was used to fit the amplitude and phase of a sinusoid with a periodicity of 3.6, and residues were designated as a part of a continuous helix if they fitted within the average error of the measurements (0.5 Hz).

The software program X-PLOR-NIH (24) was used to calculate the three-dimensional structures from the experimental data by using a basic simulated annealing protocol. An extended template was annealed against dihedral angle restraints with a 300-kcal/K force constant in the α-helical regions of the hTpoR-(479–519) peptide identified by dipolar wave fitting. As described in the rdcpot module of X-PLOR-NIH, the harmonic restraints for the 1H,15N RDCs were introduced with a linear increase of force constant from 0.01 to 7 kcal Hz−1 K−1 as the temperature was lowered to 25 K over 120 ps.
FIGURE 2. Assignments of the amide backbone and analysis of the solution NMR data. A, the hTpoR-(479–519) peptide sequence is shown. The residues of the extracellular JMR, TM domain, and cytoplasmic JMR are colored in red, blue, and black, respectively. B, the $^1$H-$^1$5N HSQC spectrum of uniformly $^1$5N-labeled hTpoR-(479–519) peptide in DHPC micelles is shown with the amide backbone assignments indicated. C, Kyte-Doolittle hydropathy plot (top), intensity plot of the amide resonances from the $^1$H-$^1$5N HSQC spectrum (middle), and the $^1$H-$^1$5N RDC plot (bottom) of the hTpoR-(479–519) peptide are shown. The seven-residue amphipathic helix is highlighted in red, and the 18-residue TM helix is highlighted in blue.
with a fixed error bound of 0.5 Hz/coupling. A P_gyr potential was applied in the calculation of the hTpoR-(479–519) peptide at a force constant of 50 kcal mol$^{-1}$ A$^{-2}$, and further optimization was performed. The accepted structures have violations of RDCs <0.5 Hz and deviation of dihedral angles <10° from those determined by dipolar wave fitting. One structural family was chosen from the accepted structures that supported the orientation of two helices as determined by solid-state NMR experiments.

RESULTS

We generated chimeric receptor constructs containing different regions of the hTpoR and hG-CSFR to identify the sequence requirements for SB394725 activity. A cellular assay based on a luciferase reporter gene construct driven by STAT response elements derived from the IRF-1 promoter (STATRE-luc) (3) was used to evaluate the activity of SB394725. The receptor constructs were co-transfected with the STATRE-luc plasmid into HepG2 cells, and the activity of SB394725 was measured (Fig. 1A). The activity of SB394725 was independent of the cytoplasmic domain sequence of hTpoR as shown by the robust activation of the chimeric receptor T(T)G, whose cytoplasmic domain consisted only of the hG-CSFR sequences, specifically the N-terminal portion with the STAT3 recruitment site. Substitution of the TM domain sequence of hTpoR with that of the hG-CSFR sequence resulted in the loss of activation of the chimeric receptor T(G)G by SB394725. These results suggested that the hTpoR TM domain is either involved in interaction with SB394725 or is required for productive conformational changes induced by binding of SB394725 to hTpoR. SB394725 activated the hG-CSFR chimeric receptor (G(T)G), which contains the hTpoR TM domain and G(13 + T)G, which contains 13 residues of the extracellular JMR and TM of the hTpoR by solution and solid-state NMR using a 41-residue hTpoR-(479–519) polypeptide, which consists of 13 extracellular JMR residues, 22 predicted TM domain residues, and 6 cytoplasmic JMR residues (Fig. 2A). The uniformly $^{15}$N-labeled hTpoR-(479–519) peptide formed a homogeneous preparation in DHP micelles (Fig. 2B) (26). The limited dispersion of frequencies in the $^{1}$H chemical shift dimension
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A.

B.

C.

D.

E.

SB394725 binding

SB394725 binding-induced
of the two-dimensional $^1$H-$^{15}$N HSQC spectrum suggests that the hTpoR-(479–519) peptide comprises primarily the α-helix, as the TM domains of type I cytokine receptors are predicted to form a single rigid α-helical structure (27). The predicted α-helix of the hTpoR TM domain starts from Ile-492 and ends at Leu-512 (Fig. 2A). Surprisingly, two distinct α-helical regions were identified from dipolar waves that mapped the RDCs measured from the in-phase and anti-phase HSQC experiments (Fig. 2B) (28, 29). The first 7-residue amphipathic helix (Thr-487 to Ser-493) may be somewhat more mobile than the second and is connected to the second 18-residue hydrophobic helix (Thr-496 to Leu-513) by a few non-helical residues. The first four predicted TM domain residues Ile-492–Val-495 were outside the second hydrophobic helix, with residues Ile-492 and Ser-493 located at the C terminus of the first amphipathic helix and residues Leu-494 and Val-495 at the interhelical region (Fig. 2C).

Solid-state NMR spectra of magnetically aligned bicelle samples were used to characterize the relative orientations of the amphipathic and hydrophobic helices of the hTpoR-(479–519) peptide. The hTpoR-(479–519) peptide was well aligned in bicelles (Fig. 3A), and the one-dimensional $^{15}$N NMR spectrum of uniformly $^{15}$N-labeled hTpoR-(479–519) peptide in the parallel bicelles (30) showed a clear separation of two distinct bands of resonances (Fig. 3A). The frequencies of the resonances observed in both one-dimensional and two-dimensional spectra were consistent with the simulated spectra (Fig. 3B). Resonances from residues in the hydrophobic TM helix occur between 160 and 200 ppm, and those between 80 and 100 ppm are associated with residues in an in-plane amphipathic helix oriented nearly parallel to the membrane surface. The resonance intensity near the isotropic frequency (120 ppm) is likely from the unstructured residues at the N and C termini of the polypeptide. The two-dimensional experimental PISEMA spectrum (Fig. 3C) of the uniformly $^{15}$N-labeled hTpoR-(479–519) peptide has resonances in the region (85–105 ppm) expected for a hydrophobic transmembrane helix with an ~28° tilt angle, which is consistent with the value predicted by the hydrophobic mismatch with the lipids (30, 31). Figure 3D is a structural model of the hTpoR-(479–519) peptide based on the NMR data shown in Figs. 2 and 3.

The two-dimensional $^1$H-$^{15}$N HSQC spectra enabled the identification of residues that are affected by binding to SB394725. The superimposed $^1$H-$^{15}$N HSQC spectra of the uniformly $^{15}$N-labeled hTpoR-(479–519) peptide with and without SB394725 are shown in Fig. 4A. Binding of SB394725 affected the resonance assigned to His-499 most significantly as shown from the chemical shift changes induced by SB394725 addition (Fig. 4B). In addition to His-499, residues Glu-484, Ser-493, Leu-494, Thr-496, Val-501, Leu-504, Ala-506, Gly-509, and Leu-511 were also affected to varying degrees upon SB394725 addition. Titration of SB394725 induced gradual changes in chemical shift of the residues affected, indicating that the changes observed are induced specifically by the hTpoR-(479–519) peptide binding to SB394725 (data not shown).

The NMR data indicated that His-499 and several residues in the extracellular JMR-TM region of hTpoR play a key role in interacting with SB394725. To further confirm the results, we performed an alanine-scanning mutagenesis study of residues in the extracellular JMR-TM region of hTpoR using the STATRE-luc reporter gene assay. Each alanine mutant receptor was tested for response to both SB394725 and recombinant hTpo. Because hTpo interacts specifically with the extracellular domain of hTpoR, alanine substitution in the extracellular JMR-TM region of hTpoR should not affect binding of hTpo to alanine mutant hTpoRs that expressed and folded properly on the cell surface. The results from alanine scanning were presented in Fig. 4, C (SB394725 treatment) and D (Tpo treatment). The residues sensitive to alanine substitution are Thr-485, Glu-488, Thr-491, Ile-492, Leu-494, Val-495, Leu-498, His-499, Leu-500, Leu-504, Val-507, and Leu-508. Alanine substitution of residues Thr-485, Glu-488, Thr-491, Leu-494, and Leu-508 affected activities of both SB394725 and hTpo, indicating that these residues were most likely involved in maintaining the expression levels and/or structural integrity of the hTpoR. Consequently, the results of alanine-scanning mutagenesis indicated that Ile-492, Leu-494, His-499, Leu-500, Leu-504, and Val-507 are the residues involved in interaction with SB394725.

Based on the NMR data, alanine-scanning results, and the physicochemical characteristics of SB394725, we concluded that residues involved in interaction with SB394725 were located at the extracellular JMR, the interhelical region, and the N terminus of the TM domain helix. A schematic diagram showing interaction between SB394725 and the hTpoR-(479–519) peptide is presented in Fig. 4E.

Because SB394725 showed strong Zn$^{2+}$-dependent activity in the luciferase reporter assay, we attempted to map Zn$^{2+}$-coordinating sites in the hTpoR-(479–519) peptide by identifying chemical shift perturbations in the two-dimensional $^1$H-$^{15}$N HSQC spectra upon Zn$^{2+}$ addition. Zn$^{2+}$ addition affected only residues Ile-492, Ser-493, and His-499 significantly (data not shown). The addition of SB394725 to the peptide-Zn$^{2+}$ complex induced additional resonance shifts, most notably Ile-492, Ser-493, Thr-496, His-499, and a few other C-terminal TM domain residues.

![Figure 4. NMR analyses of the interaction between SB394725 and the hTpoR-(479–519) peptide. A, $^1$H-$^{15}$N HSQC spectra of uniformly $^{15}$N-labeled hTpoR-(479–519) peptide without (black) and with (red) SB394725 in DHPC micelles are shown. His-499 is highlighted, and the directions of the chemical shift changes are indicated by blue arrows. B, chemical shift changes observed upon the addition of SB394725. Residues showing changes of 0.01–0.02, 0.02–0.04, and 0.04–0.1 ppm are in light blue, blue, and dark blue, respectively. Alanine substitution study results of the hTpoR-(479–519) peptide are shown in schematic diagrams showing amino acid residues that affected the activity of either SB394725 (C) or Tpo (D) treatment. The residues of helical regions with a periodicity of 3.6 residues/turn are represented in the boxes. The residues affected upon the addition of SB394725 are highlighted in the same color schemes as described for B.

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DISCUSSION

Cytokines are known to activate their respective cell-surface receptor by binding and interacting specifically with the extracellular domain of the receptor. Here we have shown that residues in the extracellular JMR-TM region of hTpoR play a crucial role in the interaction with a small molecule Tpo mimic. The unexpected role that the TM domain played in direct interaction with SB394725 and mediating hTpoR activation is consistent with previous studies suggesting that the TM domain of several cytokine receptors may be involved in ligand-independent receptor dimerization and could also play a key role in mediating receptor activation (7, 13–17).

The hTpoR extracellular JMR-TM peptide unexpectedly comprises two α-helices separated by a few non-helical residues. Kubatsky et al. reported that the first few residues of the predicted TM domain of Ep0R form α-helix cap (27). Consistent with our observation, Ile-492–Val-495 (the first four predicted TM residues of hTpoR) were outside the predicted hydrophobic TM helix. However, instead of formation of a helix cap, our NMR data show that the extracellular JMR of hTpoR contains a short seven-residue amphipathic helix and that the first two predicted TM residues Ile-492 and Ser-493 are a part of the amphipathic helix. We observed that binding of Zn$^{2+}$ and SB394725 to the hTpoR-(479–519) peptide affected the resonances of Ile-492, Ser-493, and His-499. Interestingly, cysteine substitution of residues analogous to Ile-492 and Ser-493 in mEp0R (Leu-226 and Ile-227, respectively) was reported to result in presumed disulfide bond-mediated, constitutively active mEp0R (15). The collective information suggests that binding of SB394725 to hTpoR presumably brings two extracellular JMR-TM peptides into an active orientation involving residues Ile-492 and Ser-493 and is possibly facilitated by conformational flexibility of the non-helical residues located between the two α-helices.

His-499 was identified as a key residue in determining the selective interaction between SB394725 and hTpoR as shown by mutagenesis studies and NMR experiments (Figs. 1 and 4). Interestingly, His-499 is not required by Tpo-induced TpoR activation (Fig. 4), indicating that His-499 is not an essential residue for the formation of a conformationally active dimeric receptor. Zn$^{2+}$ addition induced a selective chemical shift perturbation of His-499, an amino acid with a known propensity for metal binding. Because SB394725 showed strong Zn$^{2+}$-dependent activity, our data indicates that SB394725 presumably binds to hTpoR through Zn$^{2+}$-mediated interaction with His-499. More detailed biophysical and chemical studies will be required to determine the exact stoichiometry of the SB394725-Zn$^{2+}$-His-499 complex and the binding affinity of SB394725 to the extracellular JMR-TM peptide in the presence and absence of Zn$^{2+}$.

The NMR results showed that the addition of SB394725 induced chemical shift perturbations of the C-terminal residues of the TM domain. Because SB394725 contains a sulfonic acid moiety, it is unlikely that this charged molecule could move deeply into the highly hydrophobic TM environment. This is consistent with modeling information obtained from molecular docking of SB394725 to the hTpoR-(479–519) peptide, indicating that no significant interactions at the C terminus of the TM domain of hTpoR occurred (data not shown). Therefore, the chemical shift perturbations of the C-terminal residues Leu-504, Ala-506, Gly-509, and Leu-511–Leu-513 induced by the addition of SB394725 were most likely the result of conformational changes induced by the binding of SB394725 as opposed to direct interactions with SB394725.

Taken together, our data, for the first time, provide detailed biophysical information on ligand-induced movement of cytokine receptor extracellular JMR-TM region residues and thus offer valuable insight into both the distinctive mechanism of action of SB394725 and the general mechanism of cytokine receptor activation.

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