Mutational Analysis of Thrombopoietin for Identification of Receptor and Neutralizing Antibody Sites*

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Thrombopoietin (TPO) is the hematopoietic cytokine responsible for stimulation of megakaryocyte formation and regulation of platelet release (1–3). The gene for TPO encodes a 38-kDa protein (332 amino acids) that can be divided into two distinct domains (1, 4). The N-terminal region (153 amino acids) is predicted to be a four-helix bundle and has considerable sequence similarity to erythropoietin (EPO; 23% amino acid identity), whereas the C-terminal domain (154 amino acids) is predicted to be a four-helix bundle and has considerable sequence similarity to erythropoietin (EPO; 23% amino acid identity), whereas the C-terminal domain has no homology to known proteins and contains several asparagine-linked glycosylation sites. A number of studies have indicated that the N-terminal domain, TPO1–153, is responsible for binding and activation of the TPO receptor (MPL) (1, 5). The extracellular domain of the receptor for TPO has considerable sequence similarity to the other receptors in the class I hematopoietin receptor superfamily (6).

The four-helix bundle cytokines typically activate their receptors by homo- or hetero-oligomerization in which two distinct binding sites on a single hormone sequentially bind two of the same receptor molecules or two or more different receptor molecules, respectively (for recent review, see Ref. 7). The receptor binding sites for a number of cytokines (hGH, human prolactin, IL-4, IL-6, and EPO) map to similar regions of the hormone: one of these sites (site 1) has primary determinants in helix-4 and the loop connecting helix-1 and helix-2, whereas the other site (site 2) has primary determinants in helix-1 and helix-3. In the case of hGH, site 1 binds first followed by site 2 (8); for IL-4, site 2 appears to bind first followed by site 1 (9). We wondered if TPO contains two distinct receptor binding sites, and if so, do these map to regions seen for the other members of this class.

Alanine-scanning mutagenesis has been used to probe binding determinants in protein-protein complexes (10) (for review, see Ref. 11). Here, the sequence of TPO1–153 was aligned to that of IL-4 and mapped upon the known structure of IL-4 (12). Forty residues predicted to be solvent-exposed (mostly charged residues) were mutated to alanine and displayed as a single copy on M13 phagemid particles (13). The alanine mutants were analyzed by phage ELISA (14) for binding to the TPO receptor and to five monoclonal antibodies (mAbs) that block TPO bioactivity. Interestingly, mAbs having binding determinants in helix-4 blocked receptor binding and bioactivity, whereas those having determinants in helix-1 or helix-3 blocked bioactivity but not receptor binding. These data plus the receptor epitope mapping suggest that TPO1–153 has two receptor-binding sites that bind and activate its receptor by a sequential dimerization-type mechanism similar to that for hGH.

MATERIALS AND METHODS
Phagemid Construction, Alanine-scanning Mutagenesis, and Phage Preparation
The vector, pMP11 containing the TPO1–153 gene (gift of Dan Yansura, Genentech, Inc.), was tailored by site-directed mutagenesis (15) to create EcoRI and AvrII restriction sites that facilitate excision of the TPO gene. This was inserted into an EcoRI/XhoI-digested derivative of pHGHam-g3 (16). This construct places the TPO gene (codons 1–153) at the N-terminal end of two Gly-Gly-Gly-Ser repeats that is followed by the C-terminal domain (codons 249–406) of M13 gene III. Deletion mutagenesis (15) was used to remove codons 154, 155, and the amber stop codon (TAG) to produce the phagemid vector pML0433 to display TPO1–153. Alanine mutations in the TPO1–153 gene were made by site-directed mutagenesis (15). Monovalent phage particles were prepared

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1 The abbreviations used are: TPO, thrombopoietin; TPO1–153, the N-terminal domain of thrombopoietin; MPL, myeloproliferative leukemia virus receptor for TPO; EPO, erythropoietin; mAb, monoclonal antibody; HRP, horse radish peroxidase; ELISA, enzyme-linked immunosorbent assay; KIRA assay, kinase receptor activation assay; D, glycoprotein D; PBS, phosphate-buffered saline; TBS, tris-buffered saline; CHO, Chinese hamster ovary; BSA, bovine serum albumin; hGH, human growth hormone; IL, interleukin.
as described previously (13, 16) with the exception that the phagemid and M13K07-infected XL-1-Blue cells (Strategene, La Jolla, CA) were cultured at 30 °C. Additionally, monovalent phage particles were generated using the VCS helper phage (Strategene). Typically, phage particles obtained from 30 ml of cell culture were resuspended into 0.5 ml of 10 mM NaCl, 150 mM NaCl, pH 7.5. Material was obtained from the column with a gradient elution using ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL). Fractions containing MPL-IgG were immediately desalted using PD-10 columns (Pharmacia Biotech, Uppsala, Sweden), concentrated, and dialyzed against TBS. Concentration of MPL-IgG was estimated using $\varepsilon_{280} = 1.9$ ml mg$^{-1}$ cm$^{-1}$.

Preparation of TPO Receptor (MPL-IgG)

Recombinant MPL-IgG was prepared essentially as described by de Sauvage et al. (1). Cell culture supernatant from calcium phosphate transfectants 293 cells was passed over a protein A-agarose (IgG affinity) (Pharmacia, Cambridge, MA) column at 1 ml/min then washed extensively with 10 ml of PBS, 0.05% Tween 20, 150 mM NaCl, pH 7.5. Material was obtained from the column with a gradient elution using ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL). Fractions containing MPL-IgG were immediately desalted using PD-10 columns (Pharmacia Biotech, Uppsala, Sweden), concentrated, and dialyzed against TBS. Concentration of MPL-IgG was estimated using $\varepsilon_{280} = 1.9$ ml mg$^{-1}$ cm$^{-1}$.

Generation of Anti-TPO1–153 mAbs

mAb 1832—Balb/c mice were immunized with approximately 10 μg per dose of TPO expressed from 293 cells. TPO was formulated in QS21/alum and RIBI adjuvant (RIBI Immunocore Research Inc., Hamilton, MT) and injected intraperitoneally on weeks 1, 2, 5, 6, 9, 10, 13, 23, and 26. The immunized mice were tested for antibody response by ELISA. The mouse with the highest titer was given an additional boost of 10 μg of TPO in RIBI during week 27, and 3 days later the spleen was fused with the mouse myeloma line X63-Ag8.653 (17) using 50% polyethylene glycol 4000 (Boehringer Mannheim) by the procedure of Kohler and Milstein (18).

mAbs 1643, 1645, 1646, and 1653—Balb/c mice were immunized with approximately 20 μg of TPO in RIBI adjuvant on days 0, 3, and 21. The mice were given an additional boost of 10 μg of TPO in RIBI on day 31.

Three days later the popliteal nodes were fused with the mouse myeloma line X63-Ag8.653. The fused cells were plated in 96-well culture plates at a density of 200,000 cells per well. Hybridoma selection, using HAT media supplement (Sigma) was begun 1 day postfusion. Beginning on day 10, the hybridoma supernatants were screened for the presence of TPO1–153-specific antibodies using a solid phase ELISA. Stable antibody producing clones were obtained by limiting dilution and large quantities of specific mAbs were produced in ascites fluid. The antibodies were purified on protein A-Sepharose columns (Fementech, MD), quenched with H$_2$PO$_4$ and read by spectrophotometer at 450 nm.

TPO/Rs KIRA

A vector containing the chimeric receptor, the extracellular domain of MTL, epito-tagged with glycoprotein D (gD-MPL) and the transmembrane/intracellular domain of the receptor-type tyrosine kinase Rse (20), was transfected into CHO cells (21). The cells expressing this chimeric receptor have been shown to be responsive to TPO, as measured by intracellular tyrosine phosphorylation. Intracellular tyrosine phosphorylation was detected by incubation of the cells with biotinylated 4G10 antibody (Upstate Biotechnology Inc., Lake Placid, NY; 0.05 μg/ml), streptavidin-HRP (Sigma; 100 μl), then washed again. Wells were developed using TMB substrate (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD), quenched with H$_2$PO$_4$, and read by spectrophotometer at 450 nm.

RESULTS

Structure-based Sequence Alignment and Model of TPO—

To identify the helical core in TPO1–153 we chose to align its sequence with that of IL-4, because the structure of IL-4 is known to high resolution (12) and the four cytokines in TPO1–153 match most closely to four of the six cysteines in IL-4 (Fig. 1A). Hydrophobic residues at the i to i + 3 and/or i to i + 4 positions in the predicted helical segments of TPO1–153 align with buried residues on the IL-4 helices consistent with their having similar helical cores. Although two hydrophilic residues of TPO1–153 are predicted to be partially buried in the core (A6 and L75), IL-4 also contains hydrophilic residues at

2 M. Sadick, A. Galloway, and M. R. Mark, personal communication.
These analogous positions (Thr₁³ and Lys₁²₃) in TPO resemble members of the long-chain α-helical family. The alignment shows that α-helices 2, 3, and 4 in TPO have one to three glycine residues per helix, but glycines also occur within helices of other cytokines (28). The alignment was used as a guide for alanine-scanning (Fig. 1B).

Expression of TPO₁−₁₅₃ on Phage—TPO₁−₁₅₃ was displayed monovalently on the surface of bacteriophage as a gene III fusion (13, 16). Expression of active protein was increased by growing infected Escherichia coli at 30 °C instead of 37 °C (data not shown). The TPO₁−₁₅₃ displayed on phage bound to five anti-TPO₁−₁₅₃ mAbs with EC₅₀ values in the phage ELISA that ranged between 1 and 10 nM (data not shown). Phage particles displaying TPO₁−₁₅₃ bound specifically to immobilized TPO receptor (MPL-IgG) and the interaction was competed by soluble TPO receptor (Fig. 2). Because the amount of phage added in the competition phase of the ELISA is subsaturating, analysis of the competition curve yields an EC₅₀ value that is related to the dissociation constant (Kₐ) for the soluble receptor. The EC₅₀ value for TPO₁−₁₅₃-phage (44 ± 15 nM; Fig. 2) was almost 100-fold higher than the apparent Kₐ (~0.5 nM; data not shown) for the soluble ligand-receptor interaction. This apparent reduction in affinity is likely due to linkage to the gene III protein and others have reported similar reductions in affinity when proteins were displayed on phage. Nonetheless, the relative changes in affinity caused by alanine mutations when measured on the phage have been shown to parallel those measured on soluble proteins (14). Thus, it seems reasonable that the TPO phage ELISA can be used to compare relative affinities for various site-directed mutants of TPO.

Alanine-scanning Mutagenesis for Location of Receptor Binding Determinants—Using the alignment of TPO₁−₁₅₃ to the structure of IL-4, 40 charged and surface residues were mutated to alanine (Fig. 1B). As a control for functional display, all the alanine mutants were tested for binding to anti-TPO polyclonal antibodies (29). The sequences were aligned to best match the cysteines and regions predicted to be helical using the crystal structure of IL-4 as a guide (12). Those residues in IL-4 that are classified as consensus inner core side chains by comparison with other four-α-helical cytokines (24) are shown by asterisks. Residues in TPO that when mutated to alanine caused 2-fold or greater disruption in receptor binding are shown as bold characters with dots under them. B, residues in shaded boxes are those that were chosen for mutagenesis to alanine. Dotted lines indicate the location of the predicted helical regions in TPO₁−₁₅₃.

**FIG. 1.** A, sequence alignment of human TPO with human IL-4. The sequences were aligned to best match the cysteines and regions predicted to be helical using the crystal structure of IL-4 as a guide (12). Those residues in IL-4 that are classified as consensus inner core side chains by comparison with other four-α-helical cytokines (24) are shown by asterisks. Residues in TPO that when mutated to alanine caused 2-fold or greater disruption in receptor binding are shown as bold characters with dots under them. B, residues in shaded boxes are those that were chosen for mutagenesis to alanine. Dotted lines indicate the location of the predicted helical regions in TPO₁−₁₅₃.

3 J. T. Jones, M. D. Ballinger, J. A. Lofgren, V. D. Fitzpatrick, J. A., Wells, and M. X. Sliwkowski, manuscript in preparation.
The phage ELISA was performed as described under “Materials and Methods.” Relative EC$_{50}$ values were determined using EC$_{50} =$ 44 ± 15 nM for wild-type TPO$_{1–153}$

| TPO$_{1–153}$ mutant | EC$_{50}$ mutant/EC$_{50}$ wild type | Standard deviation$^a$ | $p$ value$^b$ |
|----------------------|-----------------------------------|------------------------|-------------|
| D8A                  | > 20$^c$                         | 1.14                   | 0.116       |
| R10A                 | 1.72                             | 0.11                   | 0.360       |
| S13A                 | 0.77                             | 0.11                   | 0.013       |
| K14A                 | 6.05                             | 1.20                   | 0.0001      |
| R17A                 | 2.54                             | 0.56                   | 0.0002      |
| D18A                 | 0.27                             | 0.03                   | 0.0055      |
| H20A                 | 0.51                             | 0.01                   | 0.057       |
| H23A                 | ND$^d$                           |                        |             |
| S24A                 | 2.07                             | 0.06                   | 0.00055     |
| R25A                 | 0.85                             | 0.07                   | 0.066       |
| S27A                 | 1.02                             | 0.06                   | 0.927       |
| Q29A                 | 0.89                             | 0.11                   | 0.067       |
| E31A                 | 1.72                             | 0.55                   | 0.0257      |
| H33A                 | 0.93                             | 0.34                   | 0.808       |
| D45A                 | 2.02                             | 0.32                   | 0.032       |
| E50A                 | 1.95                             | 1.02                   | 0.00061     |
| K59A                 | 4.04                             | 1.27                   | < 0.0001    |
| Q61A                 | 1.34                             | 0.72                   | 0.304       |
| D62A                 | 1.91                             | 1.50                   | 0.107       |
| E72A                 | 1.57                             | 1.03                   | 0.171       |
| R78A                 | ND$^d$                           |                        |             |
| S87A                 | 1.13                             | 0.03                   | 0.567       |
| S88A                 | 1.23                             | 0.19                   | 0.342       |
| Q92A                 | 1.46                             | 0.13                   | 0.071       |
| S94A                 | 1.32                             | 0.11                   | 0.18        |
| R98A                 | 3.61                             | 0.62                   | < 0.0001    |
| Q105A                | 1.74                             | 0.46                   | 0.018       |
| S106A                | 2.24                             | 0.59                   | 0.0012      |
| K117A                | 1.14                             | 0.12                   | 0.53        |
| K122A                | 1.08                             | 0.01                   | 0.699       |
| N125A                | 0.85                             | 0.18                   | 0.559       |
| L129A                | 2.63                             | 0.25                   | < 0.0001    |
| S130A                | 1.11                             | 0.09                   | 0.63        |
| Q132A                | 1.91                             | 0.04                   | 0.0019      |
| H133A                | 4.36                             | 0.08                   | < 0.0001    |
| R136A                | 5.33                             | 0.82                   | < 0.0001    |
| K138A$^e$            | > 20$^c$                         |                        |             |
| R140A                | 9.86                             | 5.00                   | 0.00011     |
| L144A                | 3.54                             | 0.67                   | < 0.0001    |

$^a$ Errors are derived from two independent trials.

$^b$ Values of $p$ were calculated using a students t-test for unpaired values (29).

$^c$ Competition of phage with soluble TPO receptor was not performed due to poor signal in the phage titration phase of the assay.

$^d$ H23A did not bind to polyclonal or monoclonal anti-TPO$_{1–153}$ antibodies. R78A did not bind with high affinity to three of the five mAbs. We could not detect binding to the TPO receptor but these mutants may have been poorly expressed so we denote them as ND, not determined.

$^e$ Only a single trial was performed.

clonal antisera (data not shown) and to a panel of five anti-TPO$_{1–153}$ mAbs (see below). All the variants bound the polyclonal antisera, except H23A which did not bind to either the polyclonal or to the five monoclonal antibodies suggesting it was either not expressed on the phage or grossly misfolded. The R78A variant bound the polyclonal antibody but did not bind with high affinity to three of the five mAbs tested. Thus, of the forty alanine mutants, we suspect that only two variants (H23A and R78A) are grossly disruptive to the structure or are not displayed on the phage.

A phage ELISA was used to test binding of the remaining thirty-eight alanine mutants to an immobilized and dimeric form of the TPO receptor (MPL-IgG). All but two variants (D8A and K138A) retained sufficient affinity for the TPO receptor to allow for competition in the assay (Table I; Fig. 3A). Very little signal was detected for D8A and K138A in the phage titration, and we estimate that their EC$_{50}$ values were at least 20-fold greater than the wild-type TPO$_{1–153}$. The second class of residues, including K14A, K52A, R136A, and R140A, had EC$_{50}$ values that ranged from 5–10-fold greater than TPO$_{1–153}$. Nine

FIG. 3. Alanine scan of TPO displayed on phage for binding to the TPO receptor. A, the EC$_{50}$ value for each alanine mutant was determined and plotted as an EC$_{50}$ ratio to wild type TPO$_{1–153}$. A value above unity indicates the alanine mutation disrupted affinity by the value shown. H23A did not bind to anti-TPO$_{1–153}$ mAbs or to polyclonal antibodies suggesting that this mutant does not express (DNE). Additionally, R78A is a mutation that disrupted binding to three mAbs and therefore may play a structural role in TPO. Errors represent standard deviations from two trials using independent TPO phage and TPO receptor preparations. For all mutants with EC$_{50}$ ratios greater than two, values of $p$ obtained using a two-tailed $t$ test (29) were less than 0.01. For some mutants (R10A, E31A, E50A, D62A, E72A, and Q105A) with EC$_{50}$ ratios between 1.5 and 2.0, the $t$ test yields $0.02 < p < 0.17$; therefore, these differences from wild-type are not statistically significant. B, TPO residues that had a significant effect on TPO receptor-binding are shown plotted on the structure of IL-4 (12). Red indicates a >20-fold disruption in affinity and these residues are labeled; dark blue indicates a 5–10-fold drop in affinity and light blue indicates a 2–5-fold loss in affinity. Because of gaps in the sequence alignment (Fig. 1A), only approximate locations are shown for some residues (Asp$^{45}$, Glu$^{50}$, Lys$^{52}$, Lys$^{138}$, and Lys$^{145}$). Because TPO is larger than IL-4 residues 1–6, 47–59, and 145–153 are not included in the model. Figs. were made using Insight II (Biosym).
other mutations (R17A, S24A, K59A, R98A, S106A, L129A, Q132A, H133A, and L144A) were found to have 2–5-fold higher EC<sub>50</sub> values than TPO<sub>1–153</sub>, and these decreases in affinity were significant (p < 0.01). Interestingly, two of the alanine mutants (D18A and H20A) showed EC<sub>50</sub> values that were two to three times lower than TPO<sub>1–153</sub>. Overall, the most functionally important receptor-binding residues are located in regions predicted to be helix-1, a loop region preceding helix-2, and along helix-3 and helix-4 (Fig. 3B).

Ala-Scan Mutagenesis for Epitope Mapping Anti-TPO<sub>1–153</sub> mAbs—Five monoclonal antibodies that bind TPO<sub>1–153</sub> (mAbs 1643, 1645, 1646, 1653, and 1831) were tested for binding to phage expressing the alanine mutations (Fig. 4A). As mentioned above, only H23A did not bind any of the mAbs, and R78A bound poorly to mAbs 1643, 1645, and 1653. The remaining 38 mutants bound to at least three of the five mAbs with near wild-type affinity suggesting that any disruptive binding effects were not the result of gross misfolding or poor expression. The dynamic range in the phage ELISA for the mAbs allows us to measure increases in EC<sub>50</sub> (decreases in affinity) of up to 20-fold. In general, only a small set of alanine mutants (one to eight) caused a >20-fold increase in EC<sub>50</sub> to any particular mAb. That only a few critical residues are important for governing mAb binding has been previously reported for a number of antibodies against several other proteins (30–33).

Mapping the disruptive alanine mutations on to the structural model of TPO shows that the epitopes for the five mAbs fall into three groups: mAbs 1643 and 1645 bind similarly, mAbs 1653 and 1831 bind similarly, and mAb 1646 is rather distinct from either of these two groups (Fig. 4B). Excluding R78A as a binding determinant, binding of mAbs 1643 and 1645 to TPO is dominated by residues on helix-3. The mutations, S87A and S94A were most disruptive to binding of mAb 1643, and S88A and Q92A and were most disruptive to binding of mAb 1645. mAbs 1653 and 1831 had primary determinants in helix-4 as well as a short segment at the N-terminal region of helix-2 (Fig. 4B). Binding of mAb 1653 was significantly effected by the mutations K52A, D62A, N125A, L129A, Q132A, H133A, R136A, K138A, and R140A. For mAb 1831, residues K59A, Q61A, D62A, and K138A (on helix-4) were found to be important for binding TPO.

Effects of alanine mutations on binding mAb 1646 were somewhat less disruptive (between 2- and 3-fold) and generally larger in number (Fig. 4A). Mutations that caused a greater than two-fold increase in the EC<sub>50</sub> for mAb 1646 were R10A, R17A, D18A, S24A, Q92A, and R98A. Generally, these mutations map to predicted helix-1 and helix-3 (Fig. 4B). Binding of phage expressing R78A could not be detected for mAb 1646. Because this residue is predicted to be located distal to the other binding determinants, it is presumed that this mutation is important for maintaining global structural integrity.

Receptor Binding and Neutralizing Activities for Anti-TPO<sub>1–153</sub> mAbs—Using surface plasmon resonance, the affinities and kinetics were measured for TPO binding to the five anti-TPO<sub>1–153</sub> mAbs (Table II). All the mAbs bound TPO with similar K<sub>d</sub> values (ranging from 1.2 to 8.3 nM) and similar kinetic parameters. Each mAb was then tested for its ability to block either direct receptor binding or activity of full-length TPO. mAbs 1653 and 1831 effectively blocked TPO binding to immobilized TPO receptor with IC<sub>50</sub> values of 1.7 and 1.3 nM, respectively, (Fig. 5A). These values were reasonably close to their respective K<sub>d</sub> values for binding to TPO. In contrast, the IC<sub>50</sub> value for mAb 1646 inhibiting binding of the TPO to its receptor was approximately 10-fold higher (13.5 nM) than its K<sub>d</sub> for binding to TPO (13.5 nM versus 1.1 nM). mAbs 1643 and 1645 only
weakly inhibited TPO receptor binding and showed only ~30% inhibition of TPO receptor binding even at a concentration of 500 nM (Fig. 5A), yet they bind TPO with $K_r$ values of 4.0 and 4.8 nM, respectively (Table II). Thus, while all five the mAbs bound with similar affinities only two effectively blocked initial binding to receptor.

A MPL/Rse KIRA assay was used to monitor TPO-induced intracellular domain phosphorylation. A receptor fusion, consisting of the extracellular domain of the TPO receptor linked to the transmembrane and intracellular domains of the Rse kinase, was transfected into CHO cells; TPO-specific tyrosine phosphorylation was measured in these cells using an ELISA-based format. All five of the mAbs were able to neutralize nearly all of the TPO-induced phosphorylation with similar affinities only two effectively blocked initial binding to receptor.

### DISCUSSION

The glycoprotein, TPO, is a key regulator of megakaryocyte and platelet production (1, 2). This molecule has tremendous therapeutic potential for patients who have undergone chemotherapy and consequently suffer from thrombocytopenia. The N-terminal domain (amino acids 1–153) is sufficient for receptor-binding and megakaryocyte proliferation (1, 5). The N-terminal domain of TPO is predicted to be a four-helix bundle, which consists of the extracellular domain of the TPO receptor linked to the transmembrane and intracellular domains of the Rse kinase; this model was used as a guide for site-directed mutagenesis to begin to elucidate the functional features of TPO.

The TPO residues that were found to affect receptor binding could be divided into three classes. The first class consists of residues that caused greater than a 20-fold increase in the $EC_{50}$ (D8A and K138A). A second class of TPO mutants increased the $EC_{50}$ ranging from between five- and fifteen-fold (K14A, K52A, R136A, and R140). A third class of mutants reduced affinity between 2- and 5-fold (R17A, S24A, K59A, R98A, S106A, L129A, Q132A, H133A, and L144A). It is likely that other residues near these also play roles in receptor binding because only charged and polar residues were targeted for mutagenesis. Without structural information, we cannot rule out the possibility that some of the alanine mutations effect receptor binding by affecting the structure of the molecule. However, we believe the vast majority of these mutations cause only minor changes in structure because thirty-eight of the forty mutants tested bound to at least three of five anti-TPO mAbs.

Generally, the side chains important receptor-binding map to one side of the predicted four helix bundle (Fig. 3B). Most of the 15 functional residues reside on helix-1 and helix-4. Seven of the 15 identified residues map onto helix-4 and four of these are positively charged side chains (His133, Arg136, Lys138, and Arg140). Most of the residues in EPO that align with the functionally important residues on helix-4 of TPO are predicted to be solvent-exposed in a model of EPO (1, 34). Lys138 in helix-4 of TPO plays a very significant role in receptor binding, yet may be involved in inner core packing of the four-a-helix bundle as predicted by our sequence alignment with IL-4 (Fig. 1A) and model (Fig. 3B). Tyr145 in EPO aligns with Lys138 of TPO (1), and a structural model of EPO (34) predicts that Tyr145 is buried in the core of the globular structure. Thus, even though the K138A mutant in TPO bound to three of the five anti-TPO mAbs, without a structure we cannot exclude the possibility that this causes local structural perturbations.

Most of the residues found to be important for receptor binding are completely conserved among humans, pigs, and mice (4). It is likely that the residues identified here for human TPO binding are also important for murine and pig TPO binding because TPO reacts broadly across these species (4). Slight variation does occur at minor binding determinants. For example, S24A which causes a 2.1-fold reduction in affinity is glycine in pig; S106 which causes a 2.2-fold reduction in affinity is aspartate in pig and glycine in mouse; His133 which causes a 2.0-fold reduction in affinity is histidine in pig and histidine in mouse. These data suggest that the residues identified in this study are important for receptor binding.

### Table II

| α-TPO, mAb | $f_{11}$ | $k_{on11}$ | $k_{off11}$ | $k_{on20}$ | $k_{off20}$ | $k_{a} \times 10^{-4}$ | $k_{b} \times 10^{-4}$ | $K_{d}$ |
|-----------|---------|------------|-------------|------------|-------------|----------------|---------------|-------|
| 1643      | 0.03    | 2.6 ± 0.3  | 8.2 ± 0.2   | 1.7 ± 0.3  | 4.8 ± 0.8   |                   |               |       |
| 1645      | 0.03    | 1.6 ± 0.3  | 8.4 ± 0.3   | 2.1 ± 0.4  | 4.0 ± 0.7   |                   |               |       |
| 1646      | 0.01    | 0.9 ± 0.4  | 3.7 ± 0.1   | 1.4 ± 0.4  | 2.6 ± 0.5   |                   |               |       |
| 1653      | 0.02    | 2.8 ± 0.1  | 8.3 ± 0.1   | 1.0 ± 0.1  | 8.3 ± 1.0   |                   |               |       |
| 1831      | 0.01    | 0.8 ± 0.2  | 2.6 ± 0.1   | 2.2 ± 0.2  | 1.2 ± 0.1   |                   |               |       |
to improve binding to the TPO receptor by 4- and 2-fold, respectively. Similarly, it was found that alanine mutations at several contact residues in helix-1 and helix-4 in hGH gave affinity improvements for hGH receptor binding (10, 28). Interestingly, these steric hindrance residues in hGH (His\(^{18}\), Phe\(^{20}\), Gln\(^{29}\), Glu\(^{65}\), and Glu\(^{174}\)) are known to be critical for hGH binding to the prolactin receptor (8) or for forming a dimeric complex that chelates Zn\(^{2+}\) (37). Thus, it is likely that Asp\(^{18}\) and His\(^{20}\) are buried at the TPO-receptor interface, and it is possible they play additional roles as yet to be determined.

Although a number of alanine mutations reduce the affinity of TPO for its receptor, most were found to have no effect (Fig. 3B). These “nonfunctional” residues are found in loops and throughout helices-1, -2, and -3. A number of these residues are involved in binding one or more of the neutralizing mAbs. Two of the antibodies (mAbs 1653 and 1831) that map to the N-terminal end of helix-2 and helix-4 are neutralizing for both receptor binding and TPO receptor activation (Fig. 5). These results suggest that this face of TPO is required for TPO receptor binding and activation, analogous to site 1 in hGH. mAbs 1643 and 1645 map to regions on helix-3 and do not block receptor binding but do inhibit receptor activation. Correlating this result to the receptor alanine-scanning data strongly suggests that residues near Ser\(^{87}\), Ser\(^{89}\), Gln\(^{92}\), and Ser\(^{94}\) are involved in binding of possibly a second TPO receptor. In addition, mAb 1646 blocked TPO activity but was only moderately effective at competing for receptor binding. This antibody maps to helix-1 and helix-3 further indicating that residues on this side of the molecule (opposite that of site 1) are required for receptor activation.

A report by Alexander et al. (38) suggests that TPO activates its receptor by homodimerization. By engineering cysteine residues into a conserved receptor dimer interface region proximal to the membrane-spanning helix in the TPO receptor, receptor mutants were found that were constitutively active in a cell-based assay. Similar findings were first reported for the EPO receptor (39). Furthermore, chimeric receptors consisting of the extracellular domains of the granulocyte colony-stimulating factor receptor (6, 40) and the hGH receptor (41) fused to the intracellular region of the TPO receptor have demonstrated that TPO receptor homodimerization precedes signal transduction.

CONCLUSIONS

Using a structural alignment and alanine-scanning mutagenesis we have begun to dissect the interactions between TPO and its receptor. As for a number of other ligand-receptor systems within the hematopoietic family (hGH, EPO, and granulocyte colony-stimulating factor receptor), our data suggest that activation of the TPO receptor occurs by a sequential homodimerization mechanism. These results provide a foundation for further analysis of TPO-receptor interactions, and suggest yet another example of how a single hormone molecule can have multiple receptor binding sites to facilitate receptor oligomerization and consequent activation.

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