Human thrombopoietin structure–function relationships: identification of functionally important residues

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Thrombopoietin (TPO) is a haematopoietic growth factor responsible for megakaryocyte progenitor proliferation and differentiation. It belongs to the four-helix-bundle cytokine family and exerts its biological effects through binding to a specific receptor, c-Mpl. With the use of site-directed mutagenesis we have generated 20 TPO mutants. Each of the TPO mutants was produced in a eukaryotic expression system and the mutants' ability to induce the proliferation of factor-dependent c-Mpl-expressing megakaryoblastic M-O7e cells was compared with that of wild-type TPO. Among the mutations studied, 10 lead to a significant decrease in TPO bioactivity. Of these ten residues, three are located in helix A of the protein (Arg¹⁰, Lys¹⁴ and Arg¹⁷) and four in helix D (His¹³³, Gln¹³², Lys¹³⁸ and Phe¹⁴¹),

indicating that in TPO, as in other cytokines, these two helices are important for functional cytokine/receptor interactions. Surprisingly, mutant Arg¹⁰ → Ala (R10A) lacked any proliferative activity, despite the fact that this mutation was recently reported to have no effect on TPO/c-Mpl binding in a TPO phage ELISA [Pearce, Potts, Presta, Bald, Fendly and Wells (1997) J. Biol. Chem. **272**, 20595–20602]. The lack of M-O7e proliferation is probably due to an inability of R10A mutant to promote receptor dimerization and thus receptor activation. Moreover we found that the Arg¹⁰ and Arg¹⁷ residues of TPO seem to be specific determinants for TPO/c-Mpl recognition. We also demonstrate that the O-glycosylation site located at position 110 of TPO is not necessary for the bioactivity of the cytokine.

INTRODUCTION

Thrombopoietin (TPO) is the primary physiological regulator of platelet production [1–3]. This cytokine has been shown to stimulate megakaryocyte progenitor proliferation and differentiation into mature megakaryocytes (reviewed in [4]). It has recently been demonstrated that TPO acts directly on, and supports, the division of cells more primitive than those committed to the megakaryocyte lineage, indicating a possible use of TPO for activation and expansion of primitive haematopoietic progenitors [5–7]. The ability of recombinant TPO to ameliorate thrombocytopenia in animal models predicts significant therapeutic potential [8,9]. TPO, or a related synthetic compound, could therefore be used to enhance the recovery of platelet production in myelosuppressed patients undergoing cancer chemotherapy.

TPO cDNAs have been recently cloned from several species, including man [1–4,10]. Sequence analyses revealed that mature human TPO is a 332-residue protein with two domains: a cytokine domain comprising the first 153 N-terminal residues and a 179-residue C-terminal domain containing multiple potential N-linked glycosylation sites. The N-terminal portion of TPO is considered to be the functional domain because it was shown to be sufficient for receptor binding, transduction of a proliferative signal in transfected cells and stimulation of megakaryocytopoiesis both *in vitro* [1,2] and *in vivo* [8,9]. This N-terminal sequence of TPO is highly conserved between species and shares 23% identity with human erythropoietin (EPO), which induces the proliferation and differentiation of immature

erythroid cells [11]. In this cytokine domain, allowing for conservative amino acid substitutions, sequence conservation approaches 50% [12]. The function of the C-terminal domain of TPO, which is not conserved between other members of this cytokine family, remains to be elucidated [13].

The tertiary structure of TPO can be inferred on the basis of sequence homology with other family members. Four conserved cysteine residues are found in the cytokine domain of TPO. Three are conserved in terms of their relative position with reference to human EPO and were found to be important for TPO activity [14–16]. There are three O-glycosylation sites located within the cytokine domain of TPO [15], which could adopt a four α -helical structure similar to that determined for growth hormone (GH), leukaemia inhibitory factor, ciliary neurotrophic factor, interleukin 6, granulocyte colony-stimulating factor and the *obese* protein leptin [17–19]. These proteins are characterized by a similar topological organization of four α -helices, with the A and D helices forming one antiparallel helical pair and the B and C helices forming another. This structure has been proposed for EPO [20] and for the other members of this cytokine family for which structural information is available (reviewed in [21]).

TPO exerts its biological effects through binding to a specific receptor expressed on the cell surface, first identified as the product of the oncogene transduced by the murine myeloproliferative leukaemia (MPL) virus [22–24]. c-Mpl, the TPO receptor, is a member of the growth hormone subfamily of class I cytokine receptors [22,25], which form a homodimeric receptor complex on ligand binding [26,27]. It has been hypothesized that

Abbreviations used: EPO, erythropoietin; GH, growth hormone; TPO, thrombopoietin.

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TPO interacts with at least two identical c-Mpl subunits to generate both proliferation and differentiation signals via the Jak/STAT signalling cascade [28]. TPO probably presents two separate interaction surfaces, one for each receptor subunit. These two surfaces have been mapped from the crystal structure of the complex of GH with two identical receptor subunits [17], and for EPO by using site-directed mutagenesis [26]. Moreover a recent study showed that most of the residues involved in TPO/c-Mpl binding were mapped to helices A and D [29]; however, this study was based on a phage ELISA and no results in terms of TPO bioactivity are available. Thus the functionally important receptor contact sites for TPO have not yet been identified.

In the present study we generated 20 TPO mutants in which the residues chosen for mutation were located mainly in the putative A and D helices. The ability of the soluble TPO mutants to induce the proliferation of factor-dependent c-Mpl-expressing megakaryoblastic M-O7e cells was compared with that of wild-type TPO. The results presented here could help in the determination of a three-dimensional model of TPO, therefore constituting a key step in the design of TPO mimetics.

MATERIALS AND METHODS

Site-directed mutagenesis

On the basis of the previously published sequence [1,2] a 0.5 kb cDNA fragment encoding both the signal peptide and the cytokine domain of TPO was amplified by reverse transcriptase-mediated PCR, with human fetal liver mRNA (Clontech, Palo Alto, CA, U.S.A.) as starting material. The PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen, Leek, The Netherlands) and sequenced (Genome Express, Grenoble, France) to ascertain the absence of any mutation.

Double-strand mutagenesis was performed with the Transformer® Site-Directed Mutagenesis kit (Clontech) as described previously [30]. Three to ten double-stranded cDNA clones from each mutagenesis reaction were screened by sequencing with the Sequenase Quick-Denature Plasmid Sequencing kit (Amersham, Les Ulis, France) and [α -³²P]dATP (Amersham). Subsequently, the entire coding sequence of each mutant was determined in order to ensure the absence of additional unwanted mutations.

Expression of wild-type and mutated TPO

HEK293 cells, which do not express TPO, were grown in minimum essential medium (Gibco-BRL, Cergy, France) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine and 100 i.u./ml penicillin/streptomycin, at 37 °C. One day before transfection, cells were seeded at a density of 3×10^6 per 56 cm² dish. Wild-type and mutated TPO cDNA species were transfected into HEK293 cells by the calcium phosphate precipitation method [31]. To ensure stable expression, G418 (Gibco-BRL) was added to the culture medium 48 h after the transfection, at a concentration of 1 mg/ml. After 2–3 weeks of selection, growing clones were isolated and transferred to 96-well plates. These clones were screened for TPO production and the producing clones were expanded.

Quantification of recombinant TPO

Transfected HEK293 cells were grown for 48–72 h in serum-free minimum essential medium, after which the culture medium was collected, centrifuged at 8400 *g* for 5 min, concentrated 10–20-fold in microconcentrators (Centricon 10, Amicon, Beverly, MA,

Figure 1 Western blot analysis and quantification of recombinant TPO

(A) Western blot detection of wild-type recombinant TPO. Increasing amounts of TPO were subjected to electrophoresis in 16% (w/v) polyacrylamide gel under reducing conditions and blotted on a Hybond®ECL® nitrocellulose membrane. TPO was detected with a rabbit polyclonal antibody (20 µg/ml), a horseradish peroxidase-conjugated anti-rabbit polyclonal antibody and the enhanced chemiluminescence detection kit as described in the Materials and methods section. The molecular masses of protein standards (MultiMark™ standard, NOVEX, San Diego) are indicated at the left. (B) Dot-blot quantification of recombinant wild-type and mutated TPO. Left panel: triplicates of increasing amounts of purified recombinant human TPO used to construct a calibration curve for the quantification experiment; right panel: duplicates of concentrated culture supernatants from cells expressing wild-type or mutated TPO. Purified recombinant human TPO and culture supernatants were spotted on the same Hybond®ECL® nitrocellulose membrane. TPO was detected with a rabbit polyclonal antibody, a horseradish peroxidase-conjugated anti-rabbit polyclonal antibody and the enhanced chemiluminescence detection kit as described in the Materials and methods section. The experiment shown is representative of three similar assays.

U.S.A.) and sterilized by filtration. For the quantification of recombinant TPO an immunoassay procedure was employed, with a purified rabbit polyclonal antibody directed against the peptide sequence QLPPQGRTTAHKDP corresponding to residues 111–124 of mature human TPO. Western blot experiments performed with this antibody gave a single band corresponding to TPO (Figure 1A). For quantification experiments (Figure 1B), concentrated culture supernatants were applied with a Bio-Dot® apparatus (Bio-Rad, Hercules, CA, U.S.A.) to a Hybond®ECL® nitrocellulose membrane (Amersham) pre-wetted in TBS (20 mM Tris/HCl/140 mM NaCl). After passive filtration of the antigen solutions, the membrane was incubated overnight

at 4 °C in a blocking solution [20 mM Tris/HCl (pH 7.5)/140 mM NaCl/5 % (w/v) non-fat dried milk]. The membrane was then incubated for 1 h at room temperature in blocking solution containing the polyclonal antibody directed against the TPO-sequence-derived peptide. After three washes in TTBS [20 mM Tris/HCl/140 mM NaCl/0.05 % (v/v) Tween 20], the membrane was incubated for 45 min at room temperature in blocking solution containing a horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Immunotech, Marseille, France). The TPO/antibodies complex was then revealed by using an enhanced chemiluminescence detection kit (Amersham), the membrane was exposed to Hyperfilm, and the signal was quantified with a densitometer apparatus (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Purified recombinant human TPO (PeproTech, Rocky Hill, NJ, U.S.A.) was used to construct a calibration curve for each quantification experiment (Figure 1B, left panel). TPO levels measured by this method correlated well with those determined by an ELISA assay developed in the laboratory (results not shown).

Proliferation assays

Concentration-dependent proliferation activities of wild-type and mutated TPO were assayed *in vitro* with the M-07e (human megakaryoblastic leukaemia) cell line [32] as previously described [33] except for the following modifications. Exponentially growing M-07e cells were seeded into 96-well plates at a concentration of 4×10^4 cells per well in 200 μ l of assay medium [RPMI 1640 with 25 mM Hepes, supplemented with 10 % (v/v) fetal bovine serum, 2 mM glutamine and 100 i.u./ml penicillin/streptomycin], supplemented with increasing amounts of conditioned medium from HEK293 cells transfected with an expression vector for wild-type or mutated TPO. The plates were incubated for 72 h at 37 °C and 1 μ Ci per well of [*methyl*- 3 H]thymidine added for 6 h. The cells were then harvested and radioactivity incorporation was determined by liquid-scintillation counting with a Tri-Carb 4640 counter (United Technologies Packard, Downers Grove, IL, U.S.A.). For each TPO mutant the proliferation assays were performed three times, each in duplicate. Concentration-response curves, $E_{\max}$ and EC_{50} values were analysed with the program ORIGIN (Microcal, Northampton, MA, U.S.A.).

RESULTS AND DISCUSSION

We designed 20 TPO mutants with the information recently published on other four-helix-bundle proteins such as EPO [20,26,34,35], prolactin [36], GH [17] and the *obese* protein leptin [19]. In a mutagenesis study of EPO, Wen et al. [35] showed that most of the functionally important receptor contact sites were localized on the predicted α -helices A and D; similar observations were reported for the GH/GH-receptor complex [17], for granulocyte/macrophage colony-stimulating factor [37] and for interleukin 2 [38]. A recent study, based on a TPO phage ELISA, indicated that most of the TPO residues involved in c-Mpl binding were located in helices A and D and a loop region between helices A and B [29].

Because the α -helices of these proteins are amphiphilic, it is probable that they pack together through their hydrophobic surfaces, which together with the disulphide bonds ensures structural stability. The biologically relevant contact sites are likely to be localized on the hydrophilic side of these molecules, as demonstrated for the solved crystal structure of the complex of GH with two receptor subunits [17] in which the hydrophilic residues of the growth factor's α -helices constitute the receptor interaction sites.

HELIX A

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tpo 1.....SPAPP--ACDLRVLSKLLRDSHVLHSRLSQCEV...32
epo 1.....APRLICDSRVLERYLLEAKEENITTGCAEH...32
gh 1.....FPTILSRLFDNAMLRRAHRLHQLAFDTYQEFEEA...34
ob 1.....VPIQVRQDDTKTLIKTIVTRINDISHTSQSVSSK...33
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HELIX D

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tpo 121.....HKDPNAIFLSFQHL...LRGKVRFLM...143
epo 130.....LRTITADTFRKLFRVYSNF.LRGKKLKYT...157
gh 158.....KNYGLLCFRKMDDKVETF.LRI.VQCRS...184
ob 104.ETLDSLGVLLEASGYSTEVVALSRLQGSLQDML...136
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Figure 2 Comparison of primary sequences for various members of the cytokine family

The analysis was restricted to cytokine residues located in the first and the fourth α -helices and their adjoining regions. Cytokine residues were aligned by the Clustal method by using the program MEGALIGN (DNASTar, Madison, WI, U.S.A.). Each sequence is numbered, starting with the first residue after the secretory signal cleavage site. Residues in α -helices are indicated in bold. The mutated TPO residues for which a significant decrease in potency was observed are indicated by stars. Abbreviations: tpo, TPO; epo, EPO; gh, GH; ob, *obese* protein leptin.

Figure 3 Schematic representation of the primary structure of TPO

The localization of the four putative α -helices of TPO is indicated in bold, in accordance with [29]. The mutated residues are indicated by stars. The bars represent the position of the deletions referred to in the Results and discussion section.

Despite the high degree of similarity between TPO and EPO, primary sequence comparison of the A and D helices revealed several significant differences (Figure 2). We hypothesized that residues shown to be important for EPO activity [35] and conserved in TPO (i.e. Lys¹⁴ and Lys¹³⁸) should also be involved in receptor binding, whereas hydrophilic residues not conserved in EPO could be critical for the specificity of the TPO/c-Mpl recognition (i.e. Arg¹⁷ and His¹³³). We therefore focused our analysis on most of the hydrophilic residues of A and D helices as well as some amino acids adjoining these regions.

The positions of the mutated residues in TPO are shown in Figure 3. Wild-type and mutated TPOs were expressed in HEK293 human cells and analysed for their ability to sustain the proliferation of TPO-responsive M-07e megakaryoblast cell line [32]. This human cell line, which constitutively expresses c-Mpl and other megakaryocyte markers [39], was chosen to avoid any species differences between TPO and its receptor in view of the differences reported for EPO/EPO receptor [35]. In addition, the M-07e cell line was also chosen because it responds strongly to TPO but not to EPO, employing the same signal transduction pathway as megakaryocytes, with the activation of Jak2 and STAT5 [40].

When stimulated by wild-type TPO, M-07e cell proliferation was concentration-dependent and saturable (Figure 4). Table 1

Figure 4 Effect of recombinant wild-type and mutated TPO proteins on M-O7e cell proliferation

Upper panel: concentration–response curves of wild-type TPO (●) and TPO mutants located on helix A: R10A (○), K14A (×), R17A (■) and S19A (□). Lower panel: Concentration–response curves of wild-type TPO (●) and TPO mutants located on helix D: Q132A (○), H133L (×), F141A (■) and K138A (□). The ability of conditioned medium from HEK293 cells expressing wild-type or mutated TPO to induce proliferation of M-O7e cells was measured by the incorporation of [³H]thymidine into DNA, as described in the Materials and methods section. Each point is the average of duplicate determinations. Results, representative of three separate experiments, are given with S.E.M. values in Table 1; 100% represents the maximal proliferating activity obtained with wild-type TPO for each experiment.

presents specific bioactivity data on all of the mutants, compared with wild-type TPO.

Effects of N- and C-terminal deletions

Two deletion mutants were constructed, Δ Ser¹Pro⁵ and Δ Val¹⁵²Arg¹⁵³, to study the involvement of the N- and C-terminal residues in TPO/c-Mpl interaction. The deleted residues were chosen such that the disulphide bond located between Cys⁷ and Cys¹⁵¹ of TPO was conserved [15] and tertiary structure was maintained.

No N-terminal deleted TPO mutant was detected in the supernatant of transfected cells (Figure 1B). Although our study did not show whether this mutant was synthesized, it is possible that the location of the deletion, downstream from the signal peptide, prevents its secretion from the cells. As a corollary to this, it has been reported that deletion of the corresponding region in EPO led to lower yields of secreted protein in comparison with the wild type, probably owing to impaired

Table 1 Proliferation data for wild-type TPO and TPO mutants

Values shown represent both $E_{\max}$ and EC_{50} ratios between mutant and simultaneously tested wild-type TPO, and are means $\pm$ S.E.M. for two to five independent determinations. $E_{\max}$ and EC_{50} were measured by non-linear regression as described in the Materials and methods section. * $P < 0.05$, ** $P < 0.01$ compared with the wild type (Student's t test). Abbreviation: n.e., not expressed.

TPO variant	$E_{\max}$ for mutant/ $E_{\max}$ for wild type	EC_{50} for mutant/ EC_{50} for wild type
Wild type	1.00	1.00
Δ S1P5	n.e.	n.e.
Δ V152R153	1.08 $\pm$ 0.07	1.58 $\pm$ 0.15*
R10A	Inactive	Inactive
S13A	0.96 $\pm$ 0.02	1.57 $\pm$ 0.41
K14A	0.41 $\pm$ 0.06**	8.38 $\pm$ 1.71*
R17A	0.70 $\pm$ 0.02*	3.62 $\pm$ 0.40*
S19A	0.96 $\pm$ 0.05	0.48 $\pm$ 0.06*
H20L	1.00 $\pm$ 0.12	1.14 $\pm$ 0.05
L71A	0.81 $\pm$ 0.02	1.33 $\pm$ 0.10
T84A	0.71 $\pm$ 0.02	5.43 $\pm$ 0.34**
Q105A	0.99 $\pm$ 0.16	12.06 $\pm$ 2.84**
T110A	0.93 $\pm$ 0.02	1.29 $\pm$ 0.17
H121L	0.83 $\pm$ 0.02	1.28 $\pm$ 0.08
K122A	0.96 $\pm$ 0.06	0.87 $\pm$ 0.06
D123N	0.93 $\pm$ 0.12	5.24 $\pm$ 0.25**
Q132A	0.88 $\pm$ 0.01	9.83 $\pm$ 2.25*
H133L	0.88 $\pm$ 0.02	10.47 $\pm$ 0.16**
R136A	0.64 $\pm$ 0.07	2.53 $\pm$ 0.73
K138A	Inactive	Inactive
F141A	0.69 $\pm$ 0.01	7.62 $\pm$ 0.95**

cleavage of the propeptide [20]. In contrast, the C-terminal TPO deletion mutant (Δ Val¹⁵²Arg¹⁵³) was readily secreted from the cells and was biologically active (Table 1). This indicates that the two non-helical C-terminal residues of the cytokine domain are not important for its binding to c-Mpl, in agreement with results reported for EPO, for which the deletion of the four last amino acids did not modify the specific activity of the protein [20].

Effects of single mutations on helix A

Within the predicted helix A, the TPO mutants that mostly affected the proliferative responses of M-O7e cells were located at positions 10 and 14 and to a smaller extent at position 17 (Table 1). Replacement of Arg¹⁰ by Ala resulted in a mutated TPO unable to promote M-O7e proliferation at concentrations of up to 50 ng/ml, whereas wild-type TPO showed its maximum efficacy at a concentration of 5 ng/ml (Figure 4 and Table 1). The mutation of this residue by Ala was recently reported to have no significant effect on TPO/c-Mpl binding [29]. These results suggest that the R10A mutant can bind efficiently to c-Mpl but is unable to effect receptor dimerization and, consequently, signal transduction. Interestingly, the same mutation was without effect in EPO [26,35], suggesting that Arg¹⁰ of predicted helix A contributes specifically to the function of TPO. An equivalent residue (Asn¹²) located at a similar position in GH (Figure 2) was found to interact with the GH receptor [17].

Replacement of Lys¹⁴ by Ala resulted in a marked attenuation of M-O7e proliferation (Figure 4 and Table 1) ($E_{\max}$ for K14A/ $E_{\max}$ for wild type = 0.41), accompanied by a significant decrease in TPO potency (EC_{50} for wild type, 0.56 ng/ml; EC_{50} for K14A, 4.69 ng/ml). Such decreases in bioactivity correlate well with the decrease in c-Mpl binding observed for this mutant [29]. In EPO, for which the corresponding residue is Arg instead

Figure 5 Helical wheel projections of the putative helices A (A) and D (B) of TPO

The projections are viewed from the NH₂ end of each helix. The hydrophobic residues are indicated by filled circles. The mutated residues for which a significant decrease in potency was observed are represented by stars.

of Lys, a similar decrease in proliferative activity was observed when mutated to Ala [26,35]. This suggests a similar pattern of interaction between the positively charged residue at position 14 of these two cytokines and their respective receptors.

Mutation R17A resulted in a 30 % impairment in the maximum proliferative response of TPO (Figure 4 and Table 1), accompanied by a 3.6-fold decrease in TPO potency (EC_{50} for wild type, 0.56 ng/ml; EC_{50} for R17A, 2.03 ng/ml). The corresponding residue in EPO is Leu, which was shown not to be involved in EPO/receptor interaction [26,35]. It is possible that Arg¹⁷ contributes to the specific TPO/c-Mpl interaction, probably owing to the longer side chain and/or the positive charge.

The three residues of helix A for which mutation affects TPO bioactivity form a cluster of positively charged residues located on the same side of the helix (Figure 5A). Their sensitivity to mutation suggests that they interact directly with the receptor, the most important being Arg¹⁰ and Lys¹⁴ of TPO. Two basic residues (Lys¹¹ and Lys¹⁵) located at a similar position in the *obese* protein leptin (Figure 2) have been recently shown to be exposed [19], and the side chains of three equivalent residues located on helix A of GH (Asn¹², Arg¹⁶ and Arg¹⁹) were found to be involved at the GH/GH receptor interface [17].

When the three other sites predicted to be on the opposite face of helix A (Ser¹³, Ser¹⁹ and His²⁰) were mutated, normal bioactivity was observed (Table 1) (Figure 5A). This side of the helix is probably not exposed to the receptor and would thus face

the hydrophobic sites of other TPO helices forming the buried core of the cytokine.

Effects of single mutations between helices A and D

Four mutations were introduced between helices A and D of TPO (Figure 3). Two (L71A and T110A) induced a response similar to that of wild-type TPO, suggesting that they are not critical for function (Table 1). The Thr residue at position 110 is a site of O-glycosylation [15], so these results demonstrate that it is not necessary for TPO function.

Mutation of Thr⁸⁴ and Gln¹⁰⁵ to Ala resulted in significant 5.4- and 12.1-fold potency decreases respectively (EC_{50} for wild type, 0.56 ng/ml; EC_{50} for T84A, 3.04 ng/ml; EC_{50} for Q105A, 6.75 ng/ml) with the maximum proliferative response remaining similar to that of the wild type (Table 1). In the absence of structural data, it is difficult to conclude whether the increased EC_{50} values observed were due to the destabilization of intramolecular bonds, or to intermolecular TPO/c-Mpl interactions. Nevertheless the fact that these two mutants induced M-O7e proliferation with comparable efficiencies to that of wild-type TPO suggests that the overall conformation of the protein is conserved.

Effects of single mutations on helix D

Five mutants were constructed in helix D of TPO and three in its upstream adjoining region (Figure 3 and Table 1). Three mutants (H121L, K122A and R136A) induced a response similar to that of wild-type TPO (Table 1).

Asp¹²³ is functionally important because, when mutated to Asn, a limited but significant decrease in TPO potency was observed (EC_{50} for wild type, 0.56 ng/ml; EC_{50} for D132N, 2.93 ng/ml), with a maximum proliferative response similar to that of the wild type (Table 1). Asp¹²³ is thought to be located in the CD loop of TPO, in the adjoining region of helix D. An Asp residue located in the corresponding region of EPO (Asp¹³⁶), was shown not to be involved in the bioactivity of the cytokine [26]. Nevertheless, with loop CD being much shorter in TPO than in EPO, its conformation and mode of interaction with c-Mpl might be different from those for the same loop in EPO.

Three other residues, located in helix D, seemed to be important for TPO activity (His¹³³, Lys¹³⁸ and Phe¹⁴¹). The K138A mutant was particularly notable in that it produced no measurable proliferation activity at a concentration up to 50 ng/ml. This effect seems to be due to an impairment of the mutant's binding of c-Mpl, as shown recently with the use of a phage ELISA [29], even if it cannot be excluded in the latter case that linkage to gene III protein of the phage leads to conformational changes. This is similar to EPO, where the corresponding K152A mutation led to a 33-fold decrease in proliferation [26]. Because this residue has been postulated to be involved in the initial EPO receptor-binding event, one could hypothesize a similar role for Lys¹³⁸ of TPO.

The mutation of His¹³³ to Leu resulted in a decrease in potency by less than 10-fold (EC_{50} for wild type, 0.56 ng/ml; EC_{50} for H133L, 5.86 ng/ml). The helical wheel projection of helix D shows that this polar residue is found in a hydrophilic environment, surrounded by Ser¹³⁰ and Arg¹⁴⁰ (Figure 5B). This suggests an accessibility of His¹³³ to solvent and thus a direct interaction between its side chain and the c-Mpl-binding site.

Mutation of Phe¹⁴¹ to Ala resulted in a 30 % decrease in M-O7e proliferation (Figure 4 and Table 1), accompanied by a 7.6-fold decrease in TPO potency (EC_{50} for wild type, 0.56 ng/ml; EC_{50} for F141A, 4.27 ng/ml). The localization of Phe¹⁴¹ on the

same side of helix D as His¹³³ and Lys¹³⁸ (Figure 5B) suggests a direct interaction with the receptor through its aromatic side chain in stacking interactions with other aromatic residues of c-Mpl.

When Gln¹³² was mutated to Ala, a significant decrease in TPO potency (EC₅₀ for wild type, 0.56 ng/ml; EC₅₀ for Q132A, 5.50 ng/ml) was observed, probably owing to the destabilization of an intramolecular bond. The topological representation of helix D shows (Figure 5B) that residue Gln¹³² is located opposite to His¹³³, Phe¹⁴¹ and Lys¹³⁸, which were proposed to be exposed to side chains of the receptor. Gln¹³² is also surrounded by hydrophobic residues (Phe¹²⁸, Leu¹²⁹, Leu¹³⁵ and Val¹³⁹), probably facing the compact core of the cytokine, and by Arg¹³⁶ residue, which is not involved in TPO bioactivity. It was recently demonstrated that the mutation of Gln¹³² to Ala was without effect on TPO/c-Mpl binding [29], indicating that this residue is probably not exposed to the receptor. The side chain of Asp¹³² is likely to be inaccessible to solvent and involved in a direct and structurally important intramolecular interaction with a carboxylic residue of TPO. Mutation of Gln¹³² to Ala probably induces the destabilization of this core steric interaction, leading to the observed decrease in TPO efficacy.

Conclusion

Ten residues on the TPO molecule have been demonstrated to be important for its bioactivity. Three of these are located in helix A of the protein and four in helix D, in good agreement with findings previously described for other cytokines such as EPO [26,35] and GH [17,41]. Our results on the functionally important residues of TPO are largely in agreement with those recently reported for the TPO residues involved in c-Mpl binding [29]. However, several mutations seem to have greater effects in terms of M-O7e cell proliferation than in the reported phage ELISA binding assay [29]. This is particularly striking for the R10A mutant, which completely lacked proliferative activity on M-O7e cells, whereas it was reported to have no significant effect in terms of binding to the c-Mpl. Such a discrepancy seems to indicate that Arg¹⁰ of TPO is a critical determinant for the c-Mpl dimerization needed for signal pathway induction.

This study gives a functional basis for the construction of a three-dimensional model of TPO and, in view of the therapeutic potential of this cytokine, might facilitate the rational design of TPO mimetics. These results might also help to identify key recognition determinants of c-Mpl.

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