

A structural model of the human thrombopoietin receptor complex

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Thrombopoietin (TPO) is a glycoprotein hormone that regulates red blood cell production. Presented here is a modeling study of the extracellular region of the human thrombopoietin receptor complex, in particular the TPO-receptor interface. The models were developed from structural homology to other cytokines and their receptors. Experimental evidence suggests that the receptor is homodimeric and it was modeled accordingly. Key interactions are shown that correlate with previous cytokine receptor complexes, and the pattern of cysteine bonding (Cys7-Cys151 and Cys29-Cys85) agrees with that experimentally determined for thrombopoietin. These models pave the way for possible mutagenesis experimentation and the design of (ant)agonists. © 1997 by Elsevier Science Inc.

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INTRODUCTION

The rate at which new protein sequences are currently being obtained far outstrips the rate of determination of their three-dimensional structures. Thrombopoietin (TPO) is one of the ever-increasing number of biologically important molecules whose structure has not yet been solved. The only simplification of this problem is the growing body of evidence suggesting that the structures of most proteins are likely to be variations on existing themes.¹

TPO is a megakaryocytic lineage-specific growth and differentiation factor.^{2,3} It acts in an analogous fashion to erythropoietin (EPO), functioning as a circulating regulator of platelet numbers. TPO has been described as possessing a two-domain structure^{4,5}: a cytokine domain (N-terminal domain) comprising the first 153 amino acids, until Arg153, followed by a larger C-terminal domain, starting at Arg154. The C-

terminal domain from position 154 to position 332 is highly glycosylated and has been shown to be unnecessary for biological activity.^{6,7} It is even speculated to be proteolytically cleaved from TPO before TPO is bound to its receptor.² The fact that in EPO, the most homologous cytokine, proteolytic cleavage occurs at Arg163 supports this.⁸ The glycosylated C-terminal domain is postulated only to assist TPO to be soluble.⁹ The N-terminal domain is highly conserved across species (80% homology between human TPO and murine TPO). It also shows 23% identity to EPO and 50% similarity with conserved substitutions³ and it has been suggested that the N-terminal domain will have a structure similar to that proposed for EPO,^{4,10} i.e., it will be a member of a subfamily of growth factors and cytokines. TPO belongs to a subgroup referred to as the hematopoietins,^{11,12} which are characterized by a four-helix bundle structure with up-up-down-down topology.¹³ TPO is predicted to belong to the long-chain hematopoietins such as human growth hormone (hGH) and EPO.^{12,14,15} There is also an alternatively spliced version of TPO with a deletion of four amino acids (positions 112–115) that is known to be inactive.¹⁶

TPO is known to bind to a receptor that belongs to the hematopoietin receptor family. These are membrane-spanning proteins, orientated with the N termini outside the cell, a single transmembrane segment, and a cytoplasmic region.¹⁷ The intracellular domains of the cytokine receptors do not show appreciable sequence homology but the extracellular domains show a much greater homology.¹⁸ The extracellular domain contains the so-called cytokine receptor motif. This motif is approximately 200 amino acids in length. It is split into two domains, each composed of seven β strands.¹⁷ Each of these domains has an immunoglobulin fold of fibronectin type III.¹⁷ The first domain has four characteristically spaced cysteine residues, and there is a WSXWS motif in the second domain near the carboxy terminal of the unit. Despite the repetition of the WSXWS motif in many cytokine receptors its role remains unclear.¹⁹ The binding interface between the cytokines and their receptors has generally been shown to be in the same area for the cytokines so far investigated.¹⁷ The D helix and AB loop constitute site 1, and site 2 is made up of the N terminus and the N-terminal end of the A helix and helix C. Each one of

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the receptor chains binds to one of these two nonidentical sites using the same region, usually from the E-F and B'-C' loops.

The thrombopoietin receptor (TPOR) is a homodimeric receptor like the human growth hormone receptor (hGHR)²⁰ and is thought to signal via the JAK STAT pathway.²¹ The crystal structure of hGH bound to the extracellular parts of its receptor remains the only structural paradigm for the hematopoietins to date. The TPOR extracellular domain is similar to the β chain of the interleukin 5 receptor (IL-5R β), IL-3R β , and the β chain of the granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR β) in that it has two repetitions of the cytokine receptor motif.²² The receptor also has an additional extracellular domain of about 50 amino acids. Thus there are unique portions of both the receptor and TPO itself. It is interesting to note that the additional domain of TPOR is hydrophilic and that the C-terminal region of TPO is highly glycosylated and thus also polar.^{4,22}

TPO is already entering pharmaceutical trials in humans for patients who have undergone chemotherapy or bone marrow transplantation that has resulted in low platelet counts.^{23,24} However, using a large protein as a drug is not ideal. Thus the design of small molecule (ant)agonists would be of great pharmaceutical interest. This is an achievable goal as the small molecule mimetic of EPO demonstrates.²⁵ Obviously to design such a mimetic a proposed structure of both the ligand and the receptor is useful. Thus the models presented here can aid in the exploration of the structure-function relationships in the TPO receptor complex and provide further insight into the mechanism and action of this potentially therapeutic cytokine.

MATERIALS AND METHODS

Alignment

Although the cytokines have similar folds their sequence homologies are generally low. EPO is the cytokine most similar to TPO, with 23% identity and 50% similarity with conserved substitutions. This means that the alignment cannot be based on amino acid identity. It was therefore based on physicochemical properties instead. As the four helix bundle has a central hydrophobic core its helices must have a hydrophobic residue every three or four residues to conserve this core. This distinctive hydrophobic pattern was used to align the TPO sequence with six other sequences of members of the cytokine family. Another feature used was the alignment of aromatic residues. Last, the possible sequence alignments were built into three-dimensional structures and examined. The alignment was performed manually using the graphical manual alignment program Cameleon.²⁶ Thus three alignments of TPO to the other cytokines were generated.

The TPOR alignment was carried out in a similar fashion based primarily on physicochemical properties. The β -sheet geometry of EPOR (whose coordinates have not yet been released) has been published.²⁵ This enabled an EPOR, hGHR alignment to be developed based on β -sheet length and location. Then the high homology of EPOR to TPOR was used to align TPOR to EPOR. The result of this procedure was an alignment of TPOR to hGHR, so that the hGHR structure²⁷ could be used as the template for the model.

The first fibronectin type III domain contains four conserved cysteine residues. In β strands A, B, C', and E these were aligned to the equivalent residues in hGHR and EPOR. The details of the π cation stacking region around the conserved WSXWS motif in EPOR and hGHR aided alignment in the second domain.²⁵ Here the π cation stacking region of hGHR was examined in three dimensions and the residues mutated to those of TPOR until a plausible π cation stacking system was built. As well as the preceding guides to the alignment, the important aromatic residues of hGHR and EPOR such as Trp104 and Trp169 of hGHR, and Phe93 of EPOR, were noted and aromatic residues from TPOR were aligned where possible.^{28,29}

Model generation

For the generation of the three-dimensional structure of TPO the Composer module implemented in Sybyl 6.1^{30,31} was used. A common C_{α} framework for the four α helices was derived from root mean square (RMS) superposition of the residues of hGH, IL-2, IL-4, and GM-CSF listed in Table 1. The fragments of the other four proteins with the highest homology to TPO were superimposed on the corresponding C_{α} framework and appropriate side-chain mutations yielded the helical core of TPO. Such a process yields the "lowest common denominator" helix length and makes manual extension of these helices reasonable. In general, long-chain cytokines have helices of 20 to 25 amino acids in length.¹⁵ This was used as a guide along with secondary structure prediction, the end of the characteristic hydrophobic pattern, and the comparative lengths of the other known structure long-chain hematopoietic cytokines. TPO is thought to be a long-chain cytokine and its loop topology was generated accordingly.

Loop searching of a database containing 347 structures including the hematopoietins used in this study then yielded the missing fragments of the protein. In the generated models of TPO the loops were constructed so that two disulfide bonds could be formed. Models of the alternatively spliced form of TPO with amino acids 112–115 deleted were then generated, by using the preferred models of TPO and replacing the CD

Table 1. Residues used to construct the framework of the helical core

Cytokine	PDB code	Residues			
		Helix A	Helix B	Helix C	Helix D
hGH	3hh	S7–L20	L75–I83	D116–M125	F166–I179
IL-2	3ink	Q11–I24	H55–L63	N88–K97	I114–S127
IL-4	1rcb	D4–L17	T44–L52	R85–A94	L109–E122
GM-CSF	1gmf	V16–S29	R58–L66	K72–A81	F103–A116

loop with a loop four residues shorter (leaving out residues 112–115).

The three-dimensional structure of TPOR was built up in much the same way, using the proposed β -sheet geometry, π cation stacking, and then working from the hGHR structure²⁷ and mutating residues in. The loops were added to this structure from a database of structures entirely of the immunoglobulin type. This specific database was chosen in order to increase the accuracy of the loop search.

The hGH complex served as a template for the generation of the possible TPO complexes, first by fitting the C_α backbone of the α -helical regions residues of each TPO model to hGH and then by fitting TPOR to hGHR via β -sheet C_α backbone fitting. The side-chain torsions of each of the models were then manually modified in order to increase favorable interactions at the interface regions.

Refinement of the receptor complexes

The refinement of the complexes is a process composed of several steps. First, after visual inspection, unfavorable side-chain interactions were manually adjusted. This was followed by 100 steps of energy minimization in the TRIPPOS force field.³² This process was repeated for the receptor and then for the complex as a whole.

The complexes were then minimized in Amber 4.1³³ with the all-atom force field for gas-phase simulations for 10 000 steps. Dynamics was carried out at 300 K for 20 ps on the side chains, then for 20 ps on the side chains and loops, followed by 20 ps with internal constraints placed on the helices and the β -sheet regions. This was followed by a free molecular dynamics run for 20 ps and then a further minimization for 10 000 steps.

However, it is worth mentioning that such runs of molecular dynamics and minimization will not correct any serious errors within the models but are intended to remove unfavorable interactions and strains within the models.

RESULTS AND DISCUSSION

Alignments

TPO was aligned with IL-2, IL4, GM-CSF, hGH, and EPO. This selection was chosen because the first four have known three-dimensional structures^{27,34–36} that can be used to build the conserved core of the molecule. EPO was selected owing to its high homology with TPO. This allows easier alignment and use of already published EPO alignments.^{5,37}

Despite the low sequence homology, this method gave a definite alignment for helices A, B, and D, and three possible alignments for helix C. All three alignments conserved the hydrophobic pattern, and gave good alignments of aromatic residues.

The three alignments were labeled TPO1, TPO2, and TPO3. On inspection of the corresponding three-dimensional models and their interactions with the receptor, TPO2 showed far fewer interactions with the receptor. In particular, TPO2 residues Arg140 and Phe141 lose potential binding sites to the receptor. Therefore this model was not refined further. The resulting alignments of TPO1 and TPO3 are shown in Figure 1.³⁹ The receptor was aligned to EPOR, hGHR, IL-2R, and IL-4R as shown in Figure 2.^{43–46}

General features of the models

The models of the TPO receptor complexes are shown in Color Plate 1.⁴⁷

TPO

The C-terminal domain of TPO was not modeled. Because TPO functions without its presence, this domain is unlikely to be of interest to the binding interface.

The helix lengths for TPO1 are Leu9–Arg25, Ala60–Ala77, Gln92–Leu108, and Asn125–Phe141. The TPO3 helices are identical except for C helix Leu90–Leu108. These helix lengths are similar. The models are different however, for if we consider the structurally conserved regions the amino acids in helix C have effectively been “wound up” in TPO3 with respect to TPO1 (see Figure 1).

These helices agree with those given in earlier work,⁴ to the extent that the helices predicted here lie within those suggested. However, when we tried to build the helices using the predicted lengths in the previous study we could not generate a particular model. In particular the C and D helices could not be connected by the 12 amino acid loop that is between them, unless the helices were moved to such an extent as to destroy the hydrophobic core.

The residues that make up the hydrophobic core of the models are given in Table 2. In TPO3 it can be seen that a charged residue Arg78 is contained within the core, but it can form a charge dipole interaction with Gln132 also within the core.

TPO1 and TPO3 both show the experimentally predicted disulfide pattern Cys7–Cys151 and Cys29–Cys85.⁴ Assuming the up–up–down–down topology of the cytokines is conserved this is the only possible disulfide combination and is a 1–4, 2–3 disulfide pattern like that shown in other cytokines.

The deleted residues (positions 112–115) of the nonfunctional alternatively spliced form of TPO are found in the CD overhand interhelical loop and not at a binding interface with the receptor. When building the models it was noted that the CD loop is rather short. It is therefore postulated that the cause of nonfunctionality is most likely to be the extra strain placed on the structure and the distortion resulting from this strain. In fact the models of this receptor complex proved to be unstable when undergoing molecular dynamics runs. The residue deletion makes the loop only 12 amino acids in length. Comparing to the most homologous cytokines, in hGH this loop is 26 amino acids in length. In the models of EPO^{37,48,49} the shortest given CD loop length is 24 amino acids.

Receptor

The N-terminal residues (Gln1 to Ser8) are not contained within these models. They correspond to the residues of hGHR whose exact positions have not been established in the X-ray structure.²⁷ In three dimensions they are situated well away from the binding site. The structure thus runs from Asp9 to Pro250. However, within this region the models do not contain the additional extracellular domain (Gln161 to Ser215). This domain is not homologous to any other known receptor structure domain and therefore cannot be modeled by homology.²² This region in the models is placed well away from the binding

IL-2	1-----APTS SSKKTQLOLEHLLL DLOM I L N G I N N Y K N P K L T R M L T-----
IL-4	1-----HK C D I T L Q E I I K T L N S L T E Q K T L C T E L T-----
hGH	1-----F P T I P L S R L F D N A M L R A H R L H Q L A F D T Y Q E F E E A Y I P K E Q K Y S F L Q
GM-CSF	1A P A R S P S P S T Q P W E H V N A I Q E A R R L L N L S -R D T A A E M N-----
EPO_H	1-----A P P R L I C D S R V L E R Y L L E A K E A E N I T G C A E H C S L N E N I T V P D T K V N-
EPO_M	1-----A P P R L I C D S R V L E R Y L L E A K E A E N V T M G C A E G P R L S E N I T V P D T K V N-
TPO1_M	1-----S P V A P A C D P R L L N K L L R D S H L L H S R L S Q C P D V D P L S I P V L L P A V D F S-
TPO1	1-----S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S-
TPO3	1-----S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S-
IL-2	42-----F K F -----Y M P K K A T E L K H L Q C L E E E L K P L E E V L N L A Q S K N F---
IL-4	29-----V T D I F ---A A S K N T T E K E T F C R A A T V L R Q F Y S H H E K D T R C L G A T A
hGH	47N P Q T S L C F S E S I P T P S N R E E T Q Q K S N L E L L R I S L L I Q S W L E P V Q F L R S V F A N S L
GM-CSF	38-----E T V -E V I S E M F D L Q E P T C I L Q T R L E L Y K Q G L R G-----
EPO_H	48-----F Y A W K R M E V G --Q Q A V E V W Q G L A L L S E A V L R G Q A L L V N S S Q-----
EPO_M	48-----F Y A W K R M E V G --E Q A V E V W Q G L S L L S E A I L Q A Q A L L A N S S Q-----
TPO1_M	48-----L G E W K T Q T E -Q S K A Q D I L G A V S L L L E G V M A A R G Q L E P S C L S S-----
TPO1	48-----L G E W K T Q M E -E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S-----
TPO3	48-----L G E W K T Q M E -E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S-----
IL-2	79-----H L R P R D L I S N I N V I V L E L K G E T T F-----
IL-4	71Q Q F H R H K Q L I R F L K R L D R N L W G L A G I N S C P V-----
hGH	102V Y G A S D S N V Y D L L K D L E E G I Q T L M G R L E D G S P R T G Q I F K Q T Y S K F D T N S H
GM-CSF	69-----S L T K L K G P L T M M A S H Y K Q H C P-----
EPO_H	87-----P W E P L Q L H V D K A V S G L R S L T T I L R A L G A Q K E A I S P P D A A S-----
EPO_M	87-----P P E T L Q L H I D K A I S G L R S L T S L L R V L G A Q K E L M S P P D T P-----
TPO1_M	89-----L L G Q L S G Q V R L L L G A L Q G L L G T-----Q L --P L Q G R T T A H K D-----
TPO1	89-----L L G Q L S G Q V R L L L G A L Q S L L G T-----Q L --P L Q G R T T A H K D-----
TPO3	89-----L L G Q L S G Q V R L L L G A L Q S L L G T -Q L --P L Q G R T T A H K D-----
IL-2	104----M C E Y A D E T A T I V E F L N R W I T F C Q S I I S T L T
IL-4	102-----K E A N Q S T L E N F L E R L K T I M R E K Y S K C S S
hGH	152N D D A L L K N Y G L L Y C F R K D M D K V E T F L R I V Q C R S V E G S C G
GM-CSF	90-P T P E T S C A T Q I I T F E S F K E N L K D F I L L V I P F D C W E P V Q E
EPO_H	127---A A P L R T I T A D T F R K L F R V Y S N F L R G K L K L Y T G E A C R T G D R
EPO_M	127---P A P L R T L T V D T F C K L F R V Y A N F L R G K L K L Y T G E V C R R G D R
TPO1_M	124-----P N A I F L S L Q Q L L R G K V R F L L L V E G P T L C V R R
TPO1	124-----P N A I F L S F Q H L L R G K V R F L M L V G G S T L C V R R
TPO3	124-----P N A I F L S F Q H L L R G K V R F L M L V G G S T L C V R R

Figure 1. Multiple sequence alignment of human TPO³⁵ with murine TPO,⁵ hGH,²⁷ IL-2,³⁸ IL-4,³⁹ GM-CSF,⁴⁰ and human and murine EPO.^{41,42} Underlined areas indicate helical residues. Consensus buried side chains are in boldface.

interface of the molecule and thus is unlikely to be involved in the binding of TPO to the receptor.

The first domain of hGHR contains three disulfide cross-links. Two of them join neighboring β strands within the same sheet, that is, A-B and F-G. The third C'-E joins the two sections of the β sandwich. The first domain of EPOR has two disulfide cross-links between sheets A-B and C'-E.

The TPO receptor model conserves the A-B and C'-E disulfide links. The modeled region of TPO contains five cysteine residues. Four are connected as described above, whereas the fifth is situated at the C-terminal end of the

additional extracellular domain aligned to Cys181 of EPOR, which does not form a disulfide link in the EPOR structure. There are three cysteine residues in the unmodeled additional extracellular domain.²²

Receptor complex

The proteins were analyzed using Procheck.⁵⁰ In both models more than 98% of residues have backbone angles that fall into commonly observed regions of the ϕ - ψ space. The peptide bonds within the structure are all transplanar with a low stan-

	A	B	C	
IL-2R	1-----AVNGTSQFT- C FYNSRANISCVWSQD-GALQ--DTSCQVHAWPDRRR---			
IL-4R	1-----MKVLQEPT- C VSDYMSISTCEWMN-GPTNC-STELRLLYQLVFLLS---			
hGHR	23 PGLKTNSSKEPKFT K CRSPERETFSCHWTDEVHHGTKNLGPIQLFYTRRNTQEWTQ			
EPOR	14 KAALLAARGPEEL- L CFTERLEDLVCFWEEA-ASAG--VGPGNYSFSYQLEDE---			
TPOR	1QDVSSLASDSEPL-KCFSRTFEDLT C FWDEEEAAPS----GTYQLLYAYPREK--			
	C'	E	F	G
IL-2R	44WNQT C ELLPVVSQA---SWACNLILGAPD-SQKLTTVDIVT--LRVLCREGVRWRV			
IL-4R	45EAHT C IPEENNGGA---GCVCHLLMDDVV-SADNYTLDLWA---GQQLLWKGSFKP			
hGHR	79EW K CPDYVSAG---ENS C YFNSSFTS-IW-IPYC I KLTS---NGGT V DEKCF			
EPOR	63PW K LCRLHQAPTARGAVRFWC S LPTADTS-SF-V P LELRVTA---ASGAPRY H RVI			
TPOR	49-PRAC P LSSQSMPHFGTRYVC Q FPDQEEVRLF-FPLHLWVK--NVFLNQTRTQRVL			
	A	B	C	
IL-2R	93MA--IQDFKPFE N RLMAPISLQVVHVETHRCNI S WEISQAS---HYFERHLEFE			
IL-4R	93SE--HV K PRA P GNLT V H-----NVSDTLLLTWSNPYPPD-NYLYNHLTYAVN			
hGHR	124SVDEIVQPD P PI A LNWT T LLNVSLTG--IHADI Q VRWEAPRNADI Q KGWMVLEY E LO			
EPOR	114HINEV V LLDAP V GLVARLADESG-----HVVLRLWP P PETPMTSH---IRYEVD			
TPOR	101FVD S VGLPAPP <i>SI</i> IKAMGGSQPG-----ELQ I SWEAPEI S ---DFLYE L R			
	C'			
IL-2R	143ARTLSPG---HTWEEAPLLTL K Q Q EW-----			
IL-4R	138IWSENDPAD F RIYNVTYL---EPSLR-----			
hGHR	178 Y KEVNET---KW K M M D P I-----LTTS-----			
EPOR	160VSAGNGAG--SV Q RVEILE---GR T E-----			
TPOR	146YGPRDPKN--STGPTV I QL---IATETCCPALQRPHSASALDQS P CAQPTMPWQD			
	E	F		
IL-2R	167-----ICLET L TPDT-----Q Y EFQ V RV K -PLQ G E F TT			
IL-4R	161-----IAAST L KSGI-----SYRAR V RAW--AQCYNTT			
hGHR	197-----VPVYSL K VD-----KEY E VR V RS K --QRNSGN			
EPOR	181-----CVLSNL R GR-----TRYTF A VR R MAEP S FG G F			
TPOR	196GP K QTSPS R EA S ALTAEGGSCL I SG L QPG-----NSYWLQL R SEPD G ISLGGS			
	G			
IL-2R	194 W SP W S Q PLAFRT K PAAL G KDT			
IL-4R	187 W SE W S P STKWHNSY R EP F EQH			
hGHR	222 Y GEF S EV L YV T LP Q MSQFT C EE D FY			
EPOR	209 W SA W S E P V SL L TP S DL D P			
TPOR	244 W GS W S L P V T V DL P GD A VAL			

Figure 2. Multiple sequence alignment of hGHR,⁴³ IL-2R,⁴⁴ IL-4R,⁴⁵ EPOR,⁴⁶ and TPOR.²² The β strands of hGHR and EPOR are indicated by the underlined sections. The conserved cysteine residues in the first domain are in boldface. The conserved WXWSWS motif in the second domain is also in boldface. The lines above the alignment indicate the names of the β strands.

dard deviation from 180° and the C_α tetrahedral distortions are low. There are no unfavorable nonbonded interactions within the models and the side-chain torsions are all within the normal range.

A Conolly⁵¹ surface area calculation shows that both of the TPO receptor complex models are compact and free of large holes. The results of the buried surface area calculations carried out with Quanta⁵² are shown in Table 3. The results show that TPO3 generally has a larger buried surface area between the proteins than does TPO1. For both TPO1 and TPO3 the buried

hydrophobic surface is larger than the buried hydrophilic surface. The buried surfaces of both TPO1 and TPO3 are comparable to those of the hGH complex, in which the hGH interface to receptor chain 1 is 1 230 Å² and to receptor chain 2 is 800 Å². The receptor chain interface is 500 Å².²⁷ However, the receptor chain interface is considerably larger than that found in the EPOR structure with the small protein mimetic; here the receptor receptor contact is only 75 Å². This may be because the EPOR chains are not bound to their natural ligand, therefore a change in relative orientation is seen.

Table 2. Residues that make up the hydrophobic core of TPO

	TPO1	TPO3
Helix A	L12, L15, S19, L22	L12, L15, S19, L22
Helix B	I63, A66, L70, V74, A77	I63, V67, L70, A76, A77, R78
Helix C	V97, L100, L104, L107	L90, L93, V97, L100, L104
Helix D	F128, F131, L135, V139	F128, F131, Q132, L135, L142
Loop	F46	L26, F46

It is notable that for both models the contact area on the side of site 1 (D helix and AB loop) is larger than that of site 2. This could imply a stepwise complex formation similar to that postulated for the hGH receptor complex.^{53,54}

However, the size of the contact surface area does not correlate well with binding free energy. In the case of the hGH-hGHR complex only a subset of 9 of the 33 interacting residues that make up the structural epitope of the receptor constitute a functional epitope. Thus only a small number of residues contribute significantly to the binding free energy.⁵⁵ This also means that the larger buried surface area of TPO3 does not automatically make it a better model.

Interactions at the binding interfaces

The interacting surfaces of the three chains of TPO3 are shown in Color Plate 2.

There are no mutation data relating to the formation of the TPO receptor complex. Therefore this analysis is based partly on analogies to other cytokines. The interactions between all of the hematopoietic cytokines and their receptors are considered to be similar. In the hGH receptor complex each receptor chain uses approximately the same region (E-F and B'-C' loops) to bind the two nonidentical regions on either side of the hGH.³³ It is postulated that these are the same regions used in IL-2, IL-4, IL-7, EPO, GM-CSF, and, in this study, the TPO-receptor complex. Thus the D helix and AB loop of TPO are region 1, binding to TPOR I, and region 2 encompasses the N terminus and the N-terminal end of the A helix together with helix C binding to TPOR II. This general similarity would be expected because the models are all based on the hGH-hGHR complex.

A selection of favorable contacts for the two models is listed

in Table 4. The interactions that are seen at each interface are discussed below.

TPO-TPOR I Arg140 of TPO is aligned with Arg178 of hGH, which is known to be important at the binding interface of the hGH receptor complex.²⁸ Arg140 is buried in a hydrophilic pocket made up of the following receptor side chains: Asp236, Glu131, and Tyr142 in TPO1. In TPO3 the pocket is considerably different and it is composed of receptor residues Ser137, Arg141, Phe139, and Asp236 (Color Plate 3). This contact appears to be important to the binding of the complex in both models.

Other residues in the D helix involved in binding are Ala126, Arg136, Ser130, and Phe141.

The AB loop of TPO also has many contacts with TPOR I. In TPO1 these include Ser47 and Thr53, which in the alignment are equivalent to hGH Leu45 and Pro61. These are both known to be important binding residues in the hGH complex.²⁸ In TPO3, only Thr53 has close contacts with the receptor. Other important residues for TPO in this region are Val39-Leu41 and Leu48-Gly49. However, one should consider that this loop is flexible and therefore able to make contacts with the receptor in other ways.

TPO-TPOR II The A helix of both models has several contacts with TPOR II. Mutagenesis studies for the other cytokines have shown that IL-4 Glu9,⁵⁶ GM-CSF Glu21,⁵⁷ and EPO Arg14⁵⁸ are all important. These residues are aligned with TPO Lys14, which in TPO1 forms a salt bridge to Glu21 of TPOR, whereas in TPO3 it forms a salt bridge to Asp236. In both models the rest of the side chain is buried within a hydrophobic patch on the receptor.

Arg24 of GM-CSF is known from mutational studies to be important for high-affinity binding to the GM-CSF receptor.⁵⁹ This residue is aligned with Arg17 of TPO. In TPO1 it can form a salt bridge to Glu131 but there is no possibility of salt bridge formation in TPO3. Asp20 of murine IL-2, which is another known binding residue,⁶⁰ is aligned with TPO Asp18, also found at the receptor-ligand interface in both models. Other important residues in the A helix are Arg10 and His23.

The second part of the binding interface to the receptor of region II consists of residues on or near helix C. The residues of helix C form many close contacts with the receptor, but it is in this area that most differences between the models can be seen. Arg98 is an important residue for binding for both the models but the binding pattern is not identical. In TPO1 it forms close contacts with receptor residues Gln73 and Asp72, whereas in TPO3 the nearby receptor residues are Tyr44, Val76, Arg77, Gln73, and Gln74 (Color Plate 4).

Table 3. Buried surface areas at the interfaces^a

	Hydrophilic		Hydrophobic		Combined	
	TPO1	TPO3	TPO1	TPO3	TPO1	TPO3
TPO/TPOR I	174	183	1 670	1 912	1 845	2 096
TPO/TPOR II	172	164	1 1346	1 674	1 517	1 838
TPOR I/TPOR II	118	188	1 039	1 388	1 156	1 575

^a In angstroms squared.

Table 4. Selected favorable contacts at the TPO–receptor complex interfaces

TPO–TPOR I ^a			TPO–TPOR II ^a			TPOR–TPOR II ^a		
TPO	TPOR I	Distance (Å)	TPO	TPOR II	Distance (Å)	TPOR I	TPOR II	Distance (Å)
TPO1								
T53 (HG1)	I136 (O)	2.1	R10 (HE)	D103 (OD2)	2.7	Y227 (HH)	Q121 (CD)	2.4
T53 (CG2)	L78 (CD2)	3.5	K14 (HZ1)	F20 (CB)	3.1	I218 (CG2)	Q121 (HE22)	2.6
H133 (HD1)	D236 (O)	2.4	K14 (HZ1)	E21 (OE2)	3.0	E124 (H)	Q126 (OE1)	2.9
H133 (NE2)	D103 (OD1)	3.3	K14 (HZ2)	E21 (OE1)	2.2	P122 (C)	Q126 (HE21)	2.6
R136 (HH12)	D236 (OD1)	2.1	K14 (HZ3)	I136 (C)	2.7	Q121 (NE2)	L144 (H)	2.6
R136 (HH22)	D236 (OD2)	3.4	R17 (HH21)	E131 (C)	3.4	Q121 (HE22)	T158 (O)	2.2
R140 (NE)	D236 (OD1)	3.4	D18 (OD2)	I136 (CG2)	3.2	V159 (H)	M117 (CE)	2.9
R140 (HH12)	E131 (OE1)	3.1	R98 (HH12)	Q73 (OE1)	2.1	S120 (HG)	V159 (O)	2.4
R140 (HH12)	F139 (C)	2.8	R98 (HH21)	D72 (OD2)	3.2			
R140 (HH21)	Y142 (CE2)	3.2						
R140 (HH2)	L140 (O)	1.9						
TPO3								
T53 (H)	F79 (CD2)	3.4	R10 (HE)	L240 (H)	2.7	Y227 (HH)	Q121 (C)	3.5
T53 (OG1)	F79 (CE2)	3.1	K14 (CA)	D236 (OD2)	3.2	Y227 (HH)	P122 (N)	2.9
N125 (HD1)	S239 (HG)	2.0	K14 (HZ1)	D103 (O)	3.0	S120 (O)	E124 (H)	2.4
H133 (ND1)	D103 (OD1)	3.3	R17 (HE)	F139 (CD1)	3.0	S219 (OG)	Q126 (HE22)	2.1
H133 (HD1)	D236 (O)	2.1	R98 (CB)	Y44 (HH)	2.9	T155 (HG1)	S128 (HG)	1.7
					R145			
H133 (CD2)	F79 (H)	3.1	R98 (NE)	R77 (HH21)	2.5	(HH12)	E130 (OE1)	1.9
R136 (HH11)	F139 (O)	3.5	R98 (CZ)	Q73 (O)	3.3	V159 (CA)	E131 (OE2)	3.4
R136 (NE)	D236 (OD2)	3.1	R98 (CZ)	E74 (O)	3.5	E124 (CD)	S219 (HG)	2.5
R140 (HH11)	F139 (O)	3.4	R98 (HH12)	V76 (CG1)	2.8	Q121 (HE21)	N225 (OD1)	2.0
R140 (HH12)	S137 (OG)	2.7						
R140 (HH12)	D236 (OD2)	2.0						

^aEntries in parentheses indicate atoms involved.

Other residues making close contacts in this region, for TPO1, are Gln92, Ser94, and Ser106; and for TPO3, residues Gly91, Gln92, Gly95, and Leu99 make close contacts.

Binding sites on the receptor

The most homologous receptor to TPOR is EPOR as previously stated. Met150 of EPOR is important in the binding of a small protein mimetic.²⁵ In this study it is aligned to Ser137 of TPOR which in TPO3 is in close contact with Arg140 of TPO on the side of TPOR I (Color Plate 2) and Lys14 of TPO on the side of TPORII. In TPO1 it is only involved in binding to Lys14 of TPO. Phe205 of EPOR is another of the binding residues to the small protein mimetic.²⁵ It is aligned to TPOR Leu240. In TPO3, Gln28 of TPO has close contacts with Leu240 of TPOR on the side of TPOR I and Arg10 is interacting on the side of TPOR II. In TPO1, there are no close contacts.

In TPO3, as we would expect from the greater buried surface area, far more sidechains of the receptor are placed in close contact to the ligand. An interesting note here is that Phe79 of TPOR which is aligned to TRP104 of hGHR and Phe93 of EPOR appears to be a very important binding residue. This agrees well with the mutation data for hGHR and EPOR which

suggest that their equivalent residues are influential in the strength of the binding of the cytokine receptor complex.^{28,29} This is further backed up by the fact that Phe93 of EPOR in the crystal structure of the complex of EPOR with the small protein mimetic has close contacts with the mimetic.²⁵

Trp169, the other aromatic residue of hGHR known to have a key role in the binding of hGH, has no equivalent in TPOR. However there is also no corresponding residue in the structure of EPOR.²⁵ Another feature of the receptor binding is that the same residues are important on both receptor chains, binding to the two non identical sides of the TPO. An example of this is Phe79. If we consider TPO1, then Phe79 in TPOR I forms part of the hydrophobic pocket for Arg10 of TPO. In TPOR II it is part of a hydrophobic patch binding to Leu48 and Ser47. In TPO3, Phe79 of TPOR II is part of a hydrophobic contact with TPO residues Ser1, Ala6 Cys7 and Arg10. In TPOR I it is in contact with TPO residues Thr53, Ser130 and His133 (Color Plate 5).

The symmetric binding could arise from the similarity of the two faces of TPO presented. For instance, site one presents a row of three basic residues: His133, Arg136 and Arg140, from along the side of the D helix. Site two also presents three basic residues to the receptor along the side of helix A: Arg10, Lys14

and Arg17. In TPO1 all six can form salt bridges to the receptor, but in TPO3 this is the case for only four.

CONCLUSIONS

Two three dimensional models of the TPO receptor complex have been generated based on homology to other hematopoietins and their receptors. Although not proven, the models appear to be reasonable in several ways. The models of TPO are 4 alpha helix bundles containing a compact hydrophobic core. There are also many similarities between interactions of the models of TPO and its receptor compared to other cytokines and their receptors. The disulfide bonds of both the receptor (Cys15–Cys25 and Cys52–Cys68) and TPO (Cys7–Cys151 and Cys29–Cys85) could be formed unambiguously and without major distortions to the structure. The disulfide bonds are identical to the experimental assignment for TPO.

The most likely places for errors in this study are within the original alignments or the docking of the TPO to TPOR. There are of course many possible binding modes, but the large size of the interfaces makes an exhaustive search of docking positions impossible. Another binding mode may be correct, particularly if the binding does not follow the hGH binding model as closely as was assumed. Yet as can be seen, the models do give rise to large buried surface areas and many favorable contacts which lend credence to the models.

The lack of mutational data on TPO makes an assessment of the two models difficult. TPO3 has a much larger buried surface area than TPO1, both between TPO and the receptor and between the two units of the receptor. In TPO3 more and closer contacts between the receptor and the ligand are suggested, particularly in the area of helix C. Calculation of the similarity scores based on the manual alignments, using the Dayhoff–mutation data matrix, show that the TPO3 alignment has a greater similarity to the other cytokines than TPO1. These facts all suggest a preference for the model TPO3. However in general the TPO1 receptor complex interactions are more like those found in other cytokine receptor complexes.

It is hoped that these models will aid the understanding of the structure function relationship within the thrombopoietin receptor complex. They also should be of great help in the designing of future mutagenesis experiments on TPO and its receptor. Results of such experiments would allow further refinement of the models and could lead to the ruling out of one of the proposed models.

ACKNOWLEDGMENTS

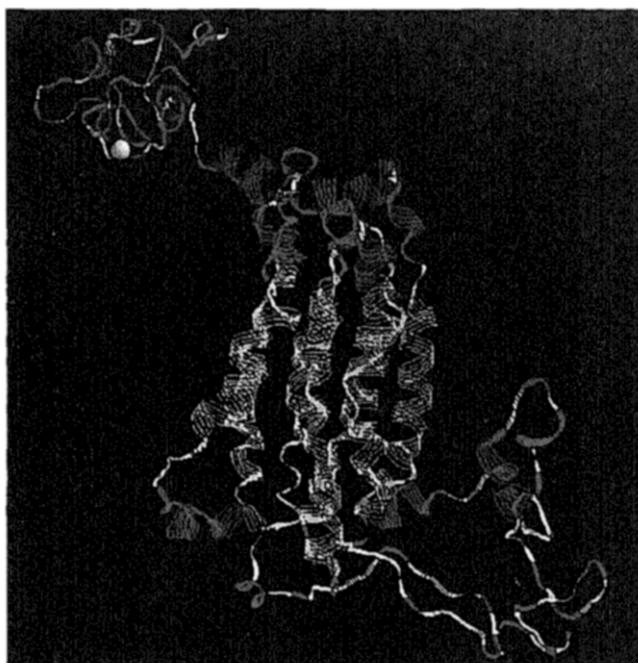
The calculations described here were performed on a Silicon Graphics Power Challenge workstation generously funded by The Wellcome Trust.

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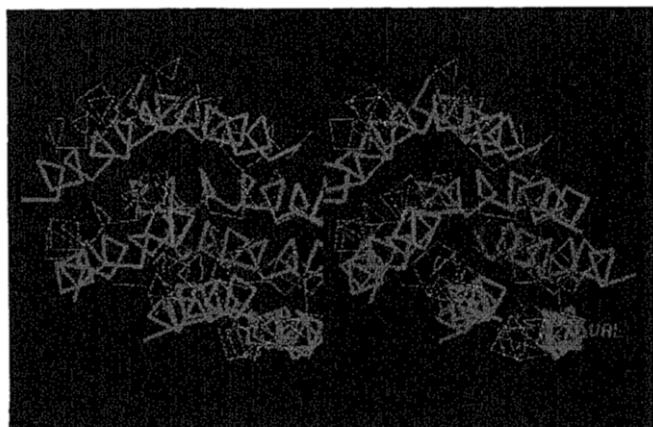
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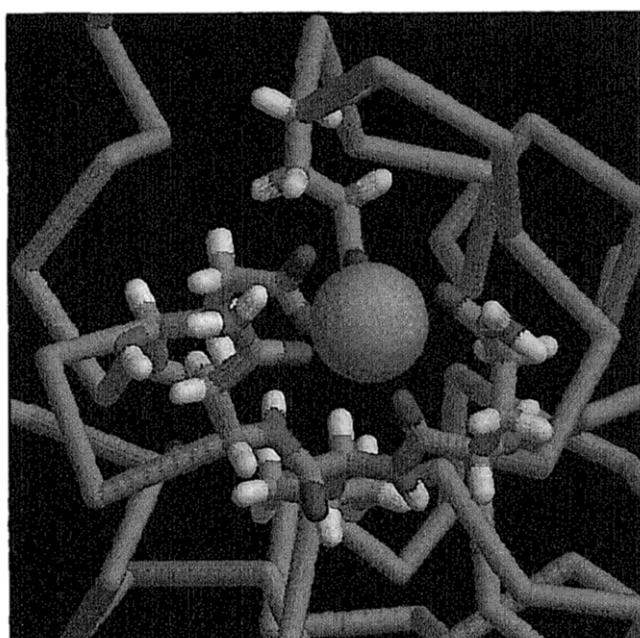
The cannabinoid receptor: Computer-aided molecular modeling and docking of ligand



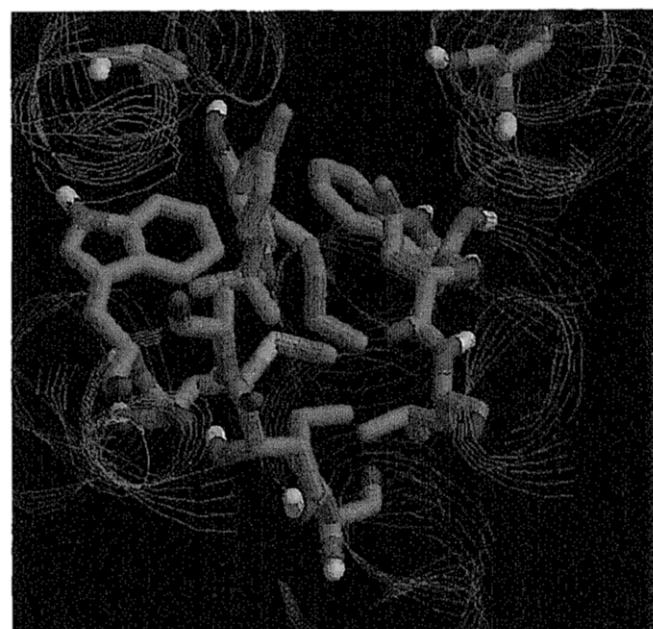
Color Plate 1. The three-dimensional modeled structure of the human cannabinoid receptor.² Transmembrane helices are shown in yellow. Extra- and intracellular helices, and turns, are shown in red and blue, respectively. Calcium is shown in white.



Color Plate 2. Stereoview of the transmembrane helices of HCNBR (green) in comparison with those of bacteriorhodopsin (blue), from the extracellular point of view. The first residue of the first helix of HCNBR (Val-121) is marked as a reference.

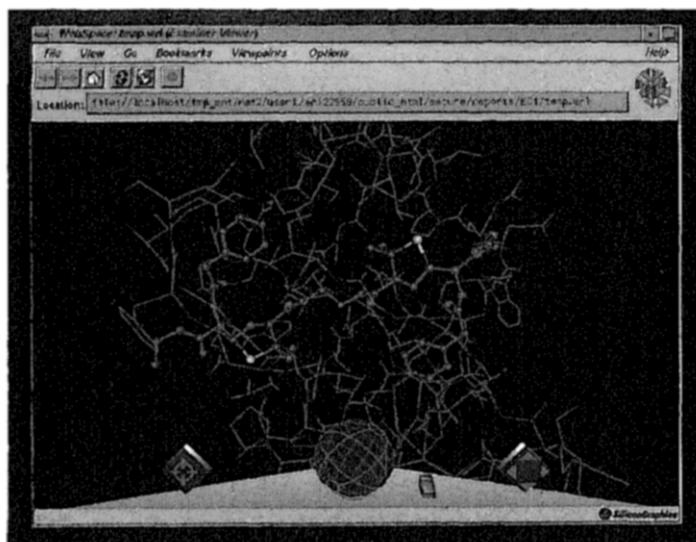


Color Plate 3. The calcium-binding cavity of the N-terminus part of HCNBR. The calcium ion binds to carboxyl groups of Glu-80, Asn-77, Gln-69, and Gln-75 as well as to the C = O group of the backbone of Gln-75.

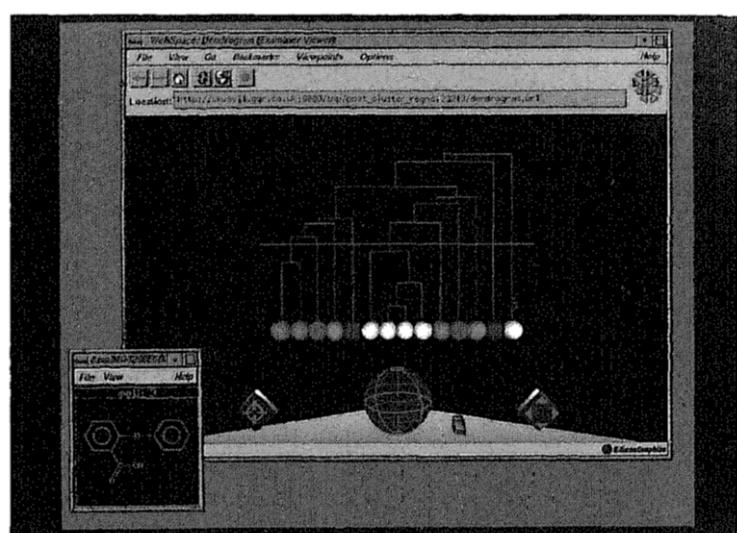


Color Plate 4. Ligand-binding site of HCNBR, d-THC shown at the vicinity of Trp-241, Met-240 (TMH-4) and Trp-356, Leu-359, Leu-360 (TMH-6). The phenolic hydroxy group of d-THC is in the hydrogen-binding vicinity of the C = O group of the backbone of Ala-198 (TMH-3).

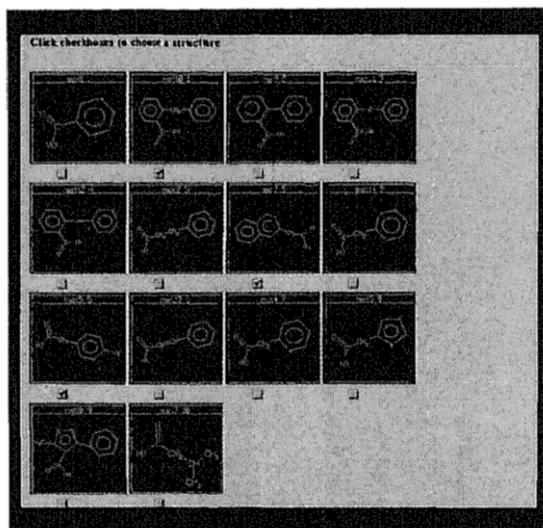
Structure-based selection of building blocks for array synthesis via the World-Wide Web



Color Plate 1. VRML representation of a penicillin-based HIV-1 protease inhibitor within the protein active site.

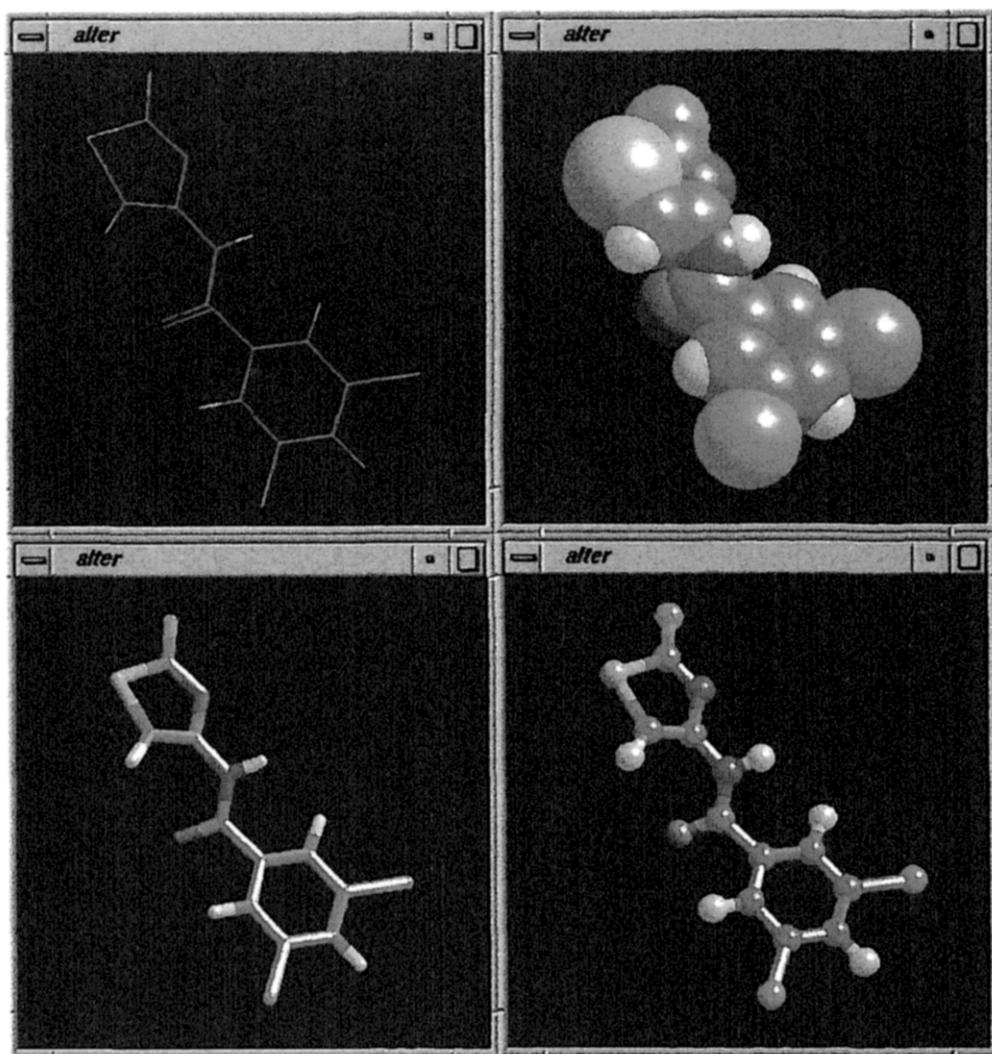


Color Plate 2. Graphical representation of dendrogram obtained from hierarchical clustering algorithm. Each of the nodes in the dendrogram is hyperlinked to a DEPICT representation of the 2D chemical drawing. The horizontal line indicates the value of the objective function selected by the user. In this case, the molecules are divided into 10 clusters.

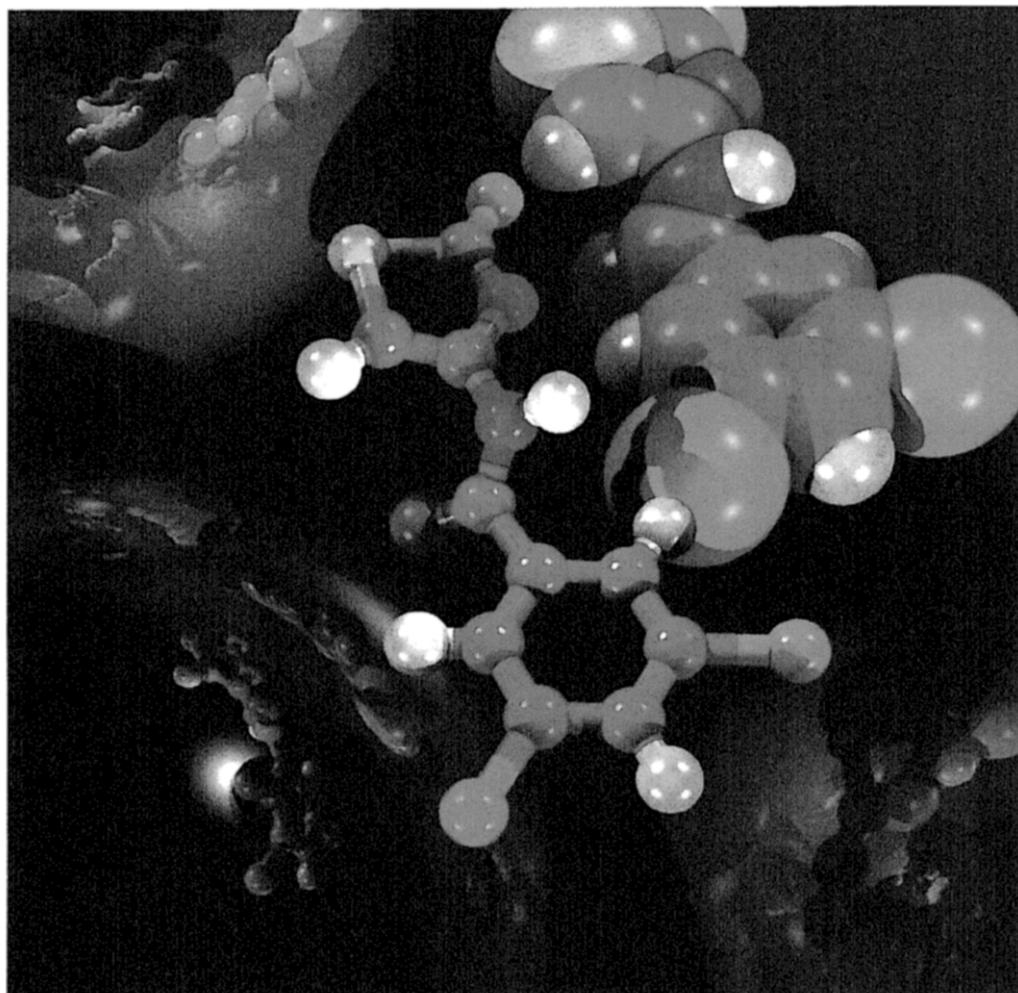


Color Plate 3. Presentation of results of cluster analysis to the user via an HTML document that enables compounds to be selected by the user for subsequent saving to disk.

ALTER: Eclectic management of molecular structure data

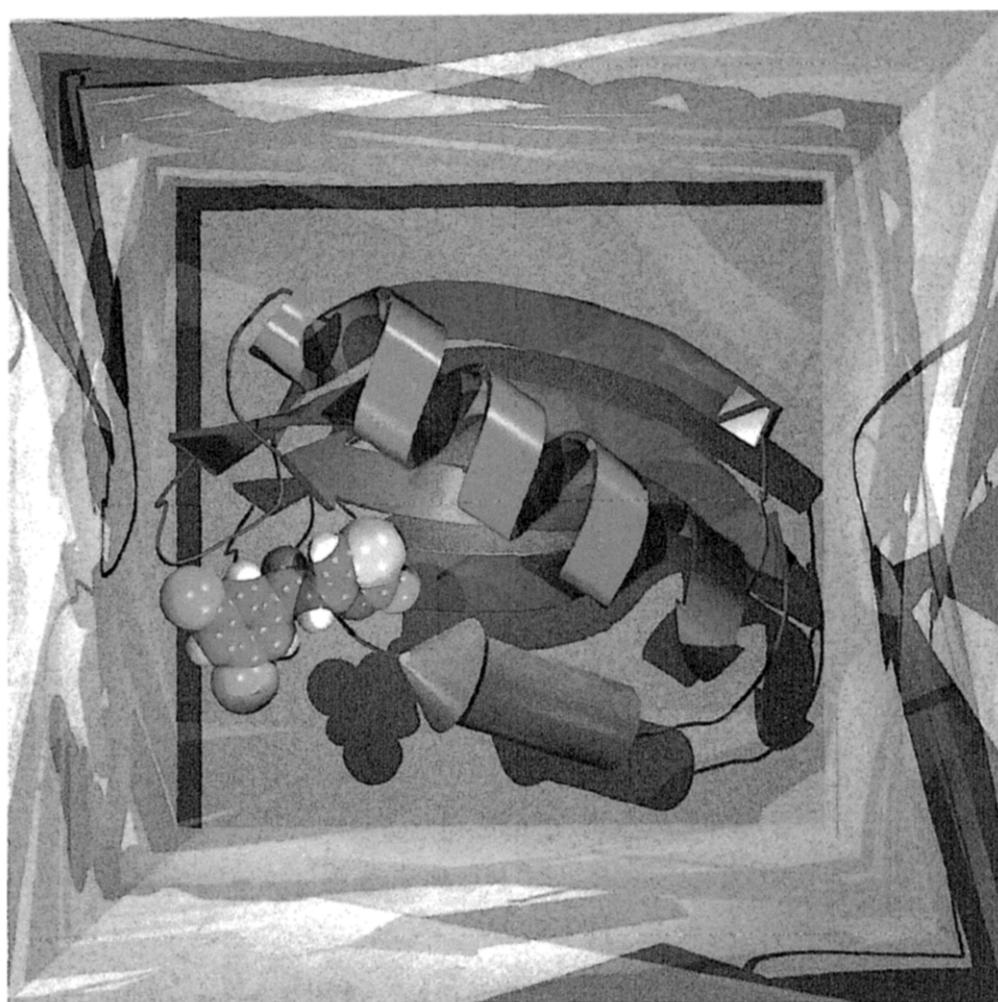


Color Plate 1. Screen shots of various display modes supported by the VIEW command. Anti-clockwise from top left: default line mode, capped stick, ball and stick, and space filling.



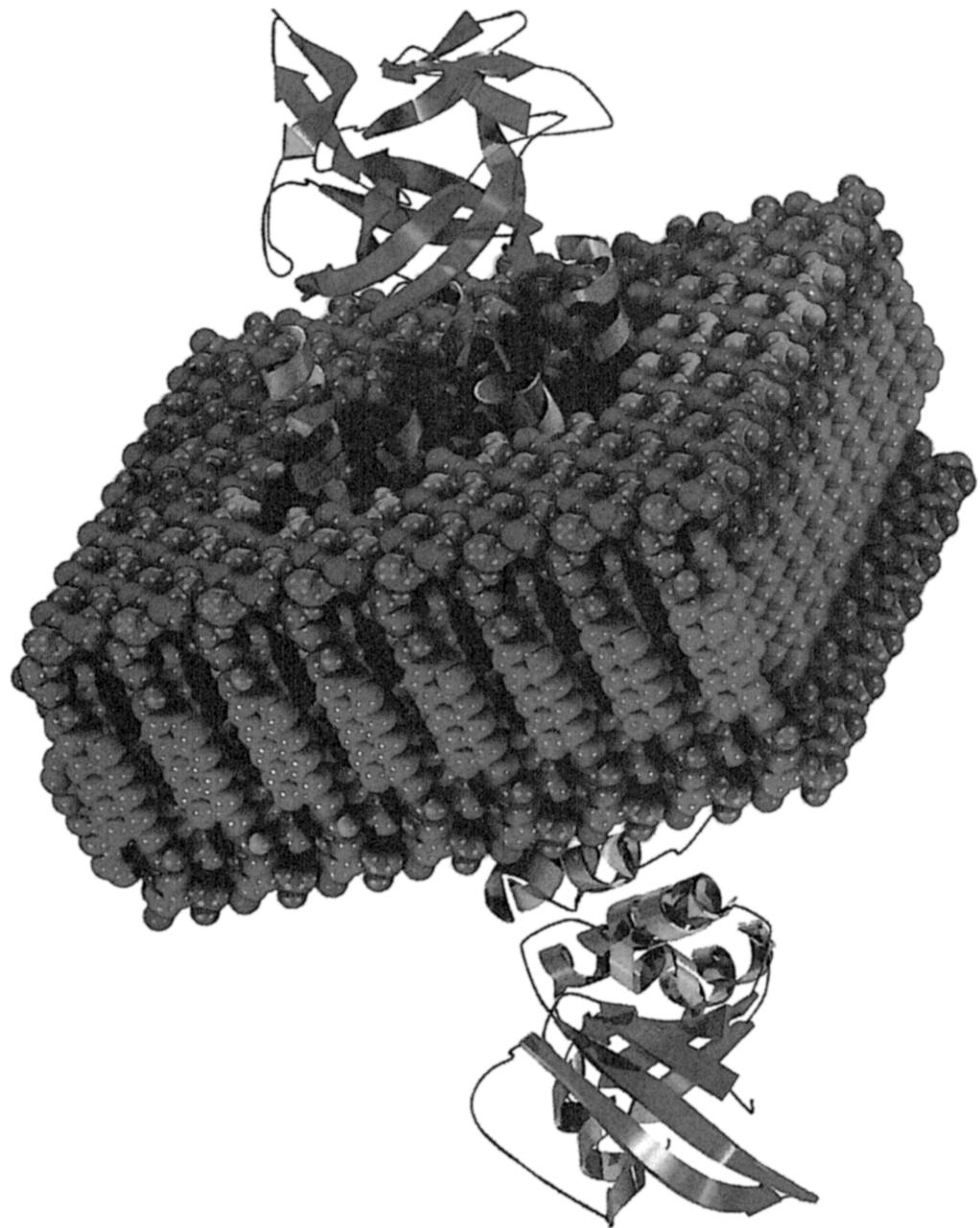
A

Color Plate 2. Various ray-traced images of molecular structures produced by POVRAY, using ALTER as an interface. (a) The small molecule from Color Plate 1, colored by atom type shown in ball-and-stick and space-filling representations. (b) A schematic depiction of a protein and its small molecule ligand. Note different helix representations and colorings. The ligand is shown atom colored and space filled. (c) A ribbon depiction of a G protein-coupled receptor model *in situ*. The GPCR sits within a lipid bilayer, is coupled to a G protein, and binds to its protein ligand.



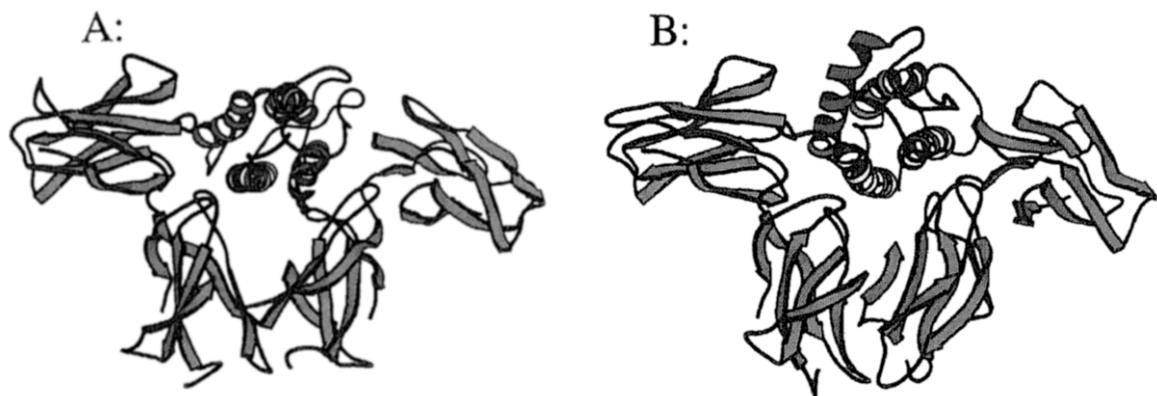
B

Color Plate 2 *Continued.*

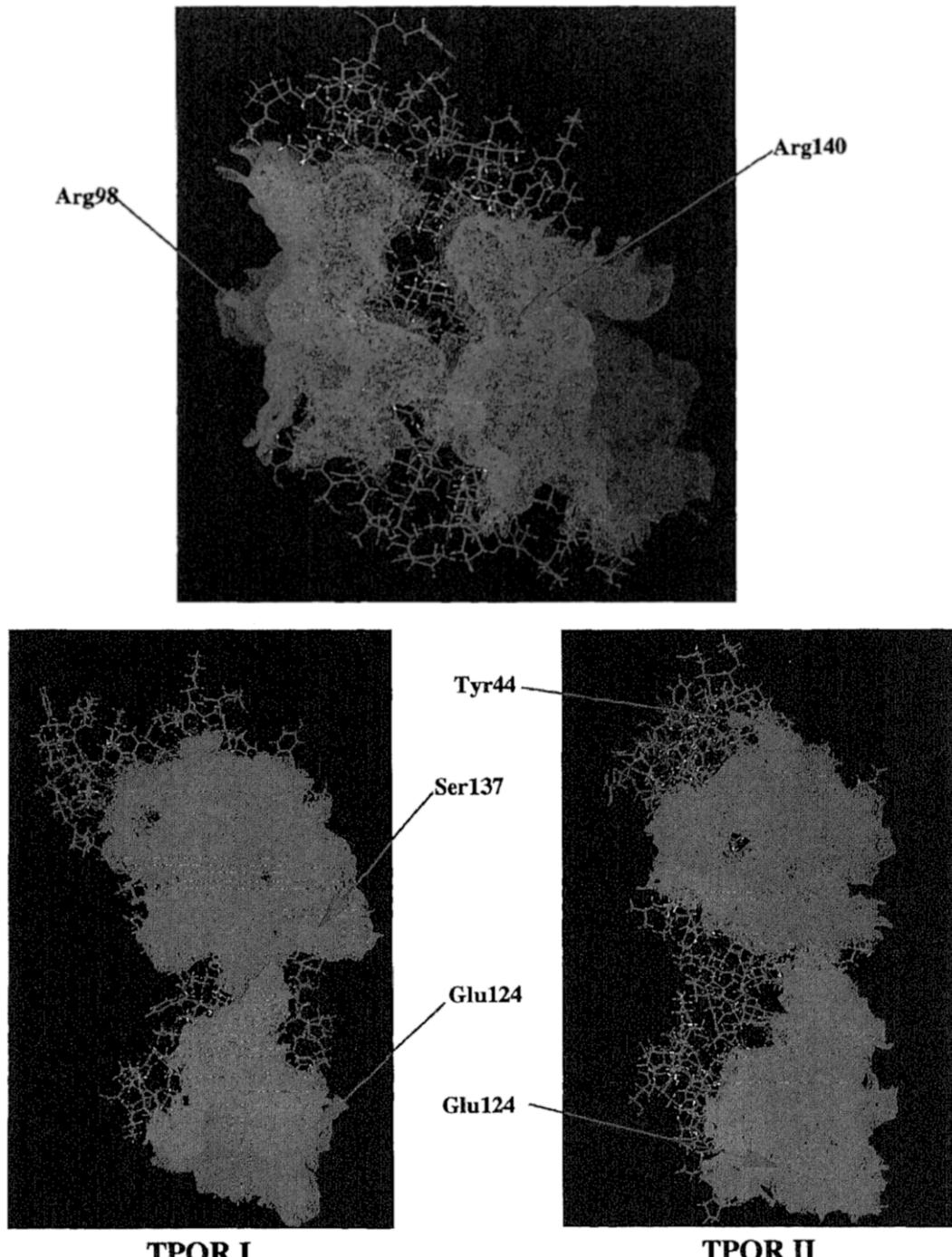


Color Plate 2 *Continued.*

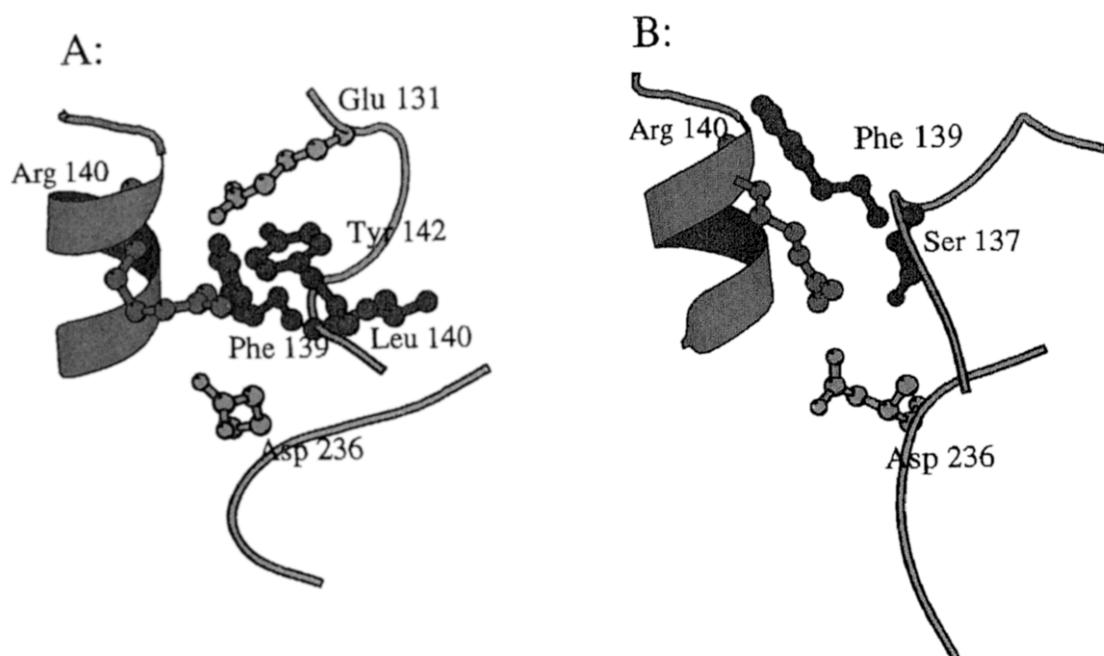
A structural model of the human thrombopoietin receptor complex



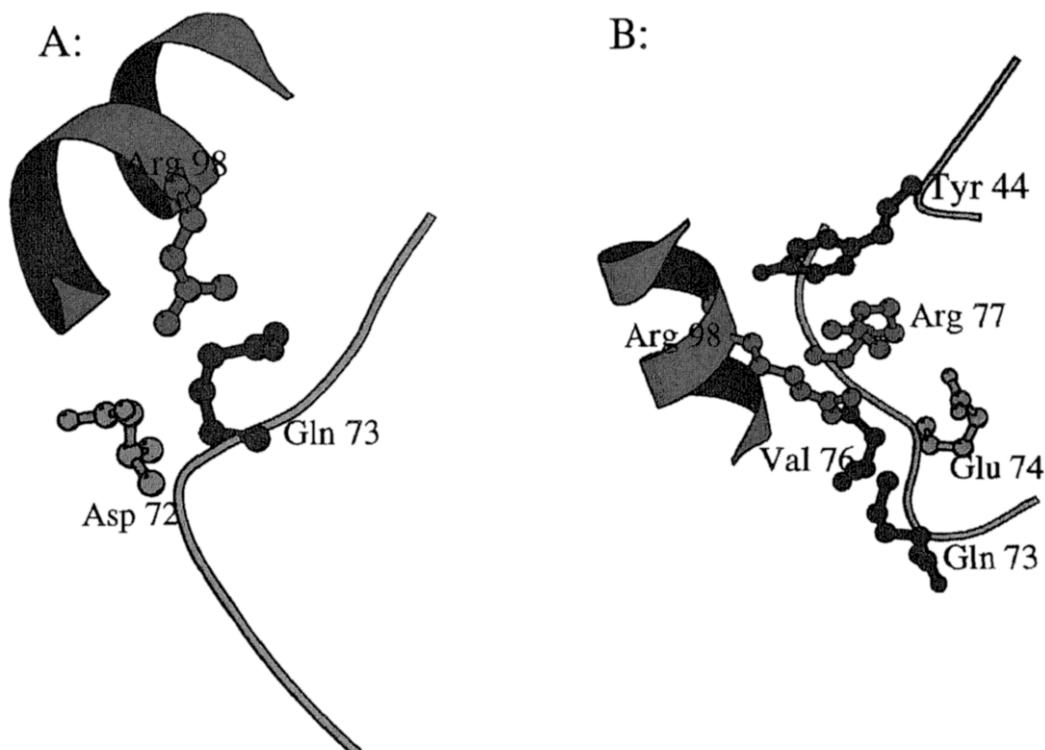
Color Plate 1. Ribbon diagrams of the TPO receptor complexes: (A) TPO1; (B) TPO3. The helices of TPO are color coded as follows: A helix, yellow; B helix, red; C helix, green; D helix, blue. Receptor chains are in green. (Picture drawn with Molscript.⁴⁷)



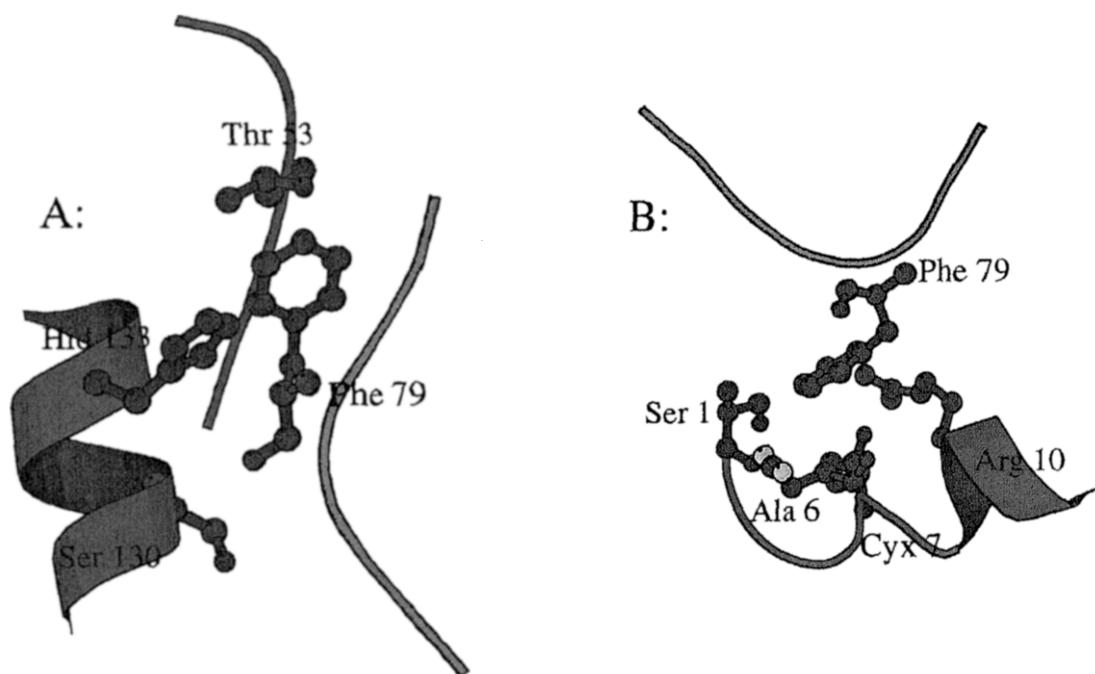
Color Plate 2. The interacting surfaces of the three chains of TPO3. Red surface shows areas between TPO and the receptor, and blue surface shows areas between the two receptor chains.



Color Plate 3. (A) TPO1; (B) TPO3. A comparison of the interaction of Arg140 of TPO with the receptor side chains. Parts of TPO (green) and TPOR (purple) are displayed schematically. Acidic residues are shown in red and basic residues are shown in blue. (Picture drawn with Molscript.⁴⁷)



Color Plate 4. (A) TPO1; (B) TPO3. A comparison of the interaction of Arg98 of TPO with the receptor side chains. Parts of TPO (green) and TPOR (purple) are displayed schematically. Acidic residues are shown in red and basic residues are shown in blue. (Picture drawn with Molscript.⁴⁷)



Color Plate 5. (A) TPOR 1; (B) TPOR II. A comparison of the interactions of Phe79 of the receptor side chains with TPO in model TPO3. Parts of TPO (green) and TPOR (purple) are displayed schematically. Basic residues are shown in blue. (Picture drawn with Molscript.⁴⁷)