PROTEINS: Structure, Function, and Genetics 27:96–109 (1997)

The Family of the IL-6-Type Cytokines: Specificity and Promiscuity of the Receptor Complexes

Joachim Grötzinger,[1]* Gunther Kurapkat,[1] Axel Wollmer,[1] Michael Kalai,[2] and Stefan Rose-John[3]
[1]*Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany;* [2]*Département de Virologie, Institut Pasteur, and Université Libre de Bruxelles, Faculté de Médecine, Laboratoire de Microbiologie, Bruxelles, Belgium;* [3]*I. Medical Clinic, University of Mainz, Section Pathophysiology, Mainz, Germany*

ABSTRACT **The cytokines IL-6, LIF, CNTF, OSM, IL-11, and CT-1 have been grouped into the family of IL-6-type cytokines, since they all require gp130 for signal transduction. Interestingly, gp130 binds directly to OSM, whereas complex formation with the other cytokines depends on additional receptor subunits. Only limited structural information on these cytokines and their receptors is available. X-ray structures have been solved for the cytokines LIF and CNTF, whose up–up–down–down four-helix bundle is common to all of these cytokines, and for the receptors of hGH and prolactin, which contain two domains with a fibronectin III-like fold. Since cocrystallization and x-ray analysis of the up to four different proteins forming the receptor complexes of the IL-6-type cytokines is unlikely to be achieved in the near future, model building based on the existing structural information is the only approach for the time being. Here we present model structures of the complexes of human and murine IL-6 with their receptors. Their validity can be deduced from the fact that published mutagenesis data and the different receptor specificity of human and murine IL-6 can be understood. It is now possible to predict the relative positions and contacts for all molecules in their respective complexes. Such information can be used for the rational design of cytokine and receptor antagonists, which may have a valuable therapeutic perspective. Proteins 27:96–109** © **1997 Wiley-Liss, Inc.**

Key words: IL-6/IL-6R complex; gp130; cytokines; model building

INTRODUCTION

Cytokines play an important role in the coordination of immune and inflammatory responses. They act on target cells via specific cell surface receptors, often consisting of different subunits for ligand binding and signal transduction.[1] Groups of cytokines have been shown to use identical signal transduction subunits, which explains their overlapping bioactivities. IL-3, IL-5, and GM-CSF share the signal transduction subunit KH97, whereas IL-6, IL-11, LIF, OSM, CT-1, and CNTF all use gp130 for signaling.[2] The latter group is called the IL-6-type cytokines[3] and belongs to the hematopoietic cytokine family, which is characterized by four antiparallel α helices (A, B, C, and D).[4,5] Other members of this family are GH, EPO, prolactin, and G-CSF. Tertiary structures are known for GH,[6] LIF,[7] CNTF,[8] and G-CSF[9,10] from x-ray analysis or NMR spectroscopy or both. For IL-6 and CNTF structural information has been obtained by homology-based model building studies validated by site-directed mutagenesis experiments.[11–15]

The extracellular parts of the cytokine receptors and their signal transduction subunits also share structural features. They all consist of a different number of domains of about 100 residues, each with a fibronectin type III-like fold. Two of these consecutive domains are called the hematopoietin receptor module and are characterized by four conserved cysteines in the first domain and a Trp-Ser-X-Trp-Ser motif in the second domain. These two domains are connected by a linker of about five residues that is proposed to serve as a hinge.[4,16] IL-6R, IL-11R, CNTFR, and the signal transducer gp130 have one such module, and the LIFR has two. For gp130 it has been shown that this module is most likely responsible for the interaction with the IL-6/IL-6R complex.[17] The cytokine receptors are called the α subunits and the signal-transducing gp130 is called the β subunit of the functional complexes, consisting of at least one IL-6, one IL-6R, and two gp130 molecules (IL-6$\alpha\beta_2$).[18] Only limited structural information on the ligand/receptor complexes of the hematopoietin family is available. The GH/GHR and GH/

Abbreviations: CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; EPO, erythropoetin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; gp130, glycoprotein 130; h, human; GH, human growth hormone; GHR, growth hormone receptor; IL-3, interleukin-3; IL-5, interleukin-5; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; IL-11, interleukin-11; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; m, murine; OSM, oncostatin M; rms, root mean square deviation.

*Correspondence to: Dr. J. Grötzinger, Institut für Biochemie, RWTH-Aachen, Pauwelsstrasse 30, D52057 Aachen, Germany. E-mail: achin@cionm1.biochem.twth-aachen.dc.

Received 11 January 1996; accepted 22 April 1996.

prolactin receptor complexes are the only members for which the structure has been solved by x-ray crystallography.[6,19] There are two receptor binding sites on the GH molecule. The higher affinity site I involves the AB loop and the C-terminal part of helix D; site II is formed by residues from helix A and helix C. The generation of the biologically active GH-$(GHR)_2$ complex proceeds through the association of an initial GH/GHR complex with a second GHR molecule that is functionally analogous to the IL-6/IL-6R/gp130 system. Therefore the high-affinity site I of GH can be regarded as the corresponding IL-6R binding site in the IL-6 molecule. Consequently, site II can be regarded as a contact site for one gp130 molecule.[20]

The observation that hIL-6 binds to the mIL-6R but mIL-6 does not bind to the hIL-6R[21] led to the construction of chimeric molecules and to the discovery of a region in hIL-6 comprising the initial part of the long loop between the A and the B helix, which is involved in signal initiation by gp130.[11] This region is in close proximity to a site that has been mapped by using neutralizing monoclonal antibodies and seems to contact the gp130 protein.[22] The analogous region is not involved in the formation of the GH/$(GHR)_2$ complex. Recent results of site-directed mutagenesis experiments have shown that this region is involved in IL-6R-dependent binding of IL-6 to gp130.[11,23,24] No detailed structural information is available on IL-6, IL-6R, and gp130, nor on the complex of these molecules. In this molecular modeling study we present models for the h- and mIL-6/IL-6R complexes. The human model complex is in excellent agreement with published results obtained by site-directed mutagenesis[11,12,23,25–35] or peptide-scanning experiments.[36] Comparison of the human and murine model complexes have enabled us to explain the species specificity of mIL-6. Based on these experimentally validated models, we discuss the possible location and orientation of the second gp130 molecule included in the complex, which transduces the signal into the cell. In addition, the differences between our model and the hexameric IL-6 receptor complex model suggested by Ward and colleagues[37] and Paonessa and colleagues[20] are discussed. An understanding of the IL-6 receptor complex will lead to the design of new experiments to elucidate the structure of this complex and its functionality that is representative for the whole family of the IL-6-type cytokines.

MATERIALS AND METHODS

The methods used for the homology-based modeling of the hIL-6 and mIL-6 structures, using G-CSF[9] (Protein Data Bank accession number: 1RHG) as a template, are described in a previous article.[11] The IL-6R and gp130 cytokine-binding domains were built as follows: The sequential alignments between IL-6R and gp130, respectively, and GHR were generated with the Husar program package.[38] Because of the poor sequence homology between these molecules the alignment was further evaluated by calculating the solvent accessibilities for all amino acids of the GHR. Residues that are hydrophobic and not accessible by the solvent have been regarded as crucial for the hydrophobic core of this protein. The alignment was then changed by shifting hydrophobic amino acids of the IL-6R and gp130, respectively, to these crucial positions. In addition, a secondary structure prediction[39] was performed for IL-6R and gp130 to see whether the predicted β strands were correctly aligned with the β strands of the GHR structure. This method, together with the known pattern of hydrophobic and hydrophilic residues in β strands, finally led to the sequential alignment shown in Figure 5B.

According to this alignment, amino acid residues were exchanged in the template. Insertions and deletions in IL-6R and gp130 were modeled by using a database search approach included in the software package WHATIF.[40] The database was searched for a peptide sequence of the appropriate length, which was fitted to the template. All loops were selected from the database so as to give a minimum rms distance between the ends of the loops and the sheets. Loops with unfavorable backbone angles or van der Waals clashes were excluded. The same approach was used to build the loops not resolved in the GH/GHR x-ray structure. Although these parts are only vaguely defined, they were chosen to satisfy general principles of protein architecture.

Finally, these model structures were energy-minimized, using the steepest descent algorithm implemented in the GROMOS[41] force field.

To create the h- and mIL-6/IL-6R complexes, the corresponding IL-6 and IL-6R molecules were oriented to each other by using the GH/GHR complex as a template. In a first step, the IL-6 molecule was fitted onto the GH structure (using only the Cα positions of the helices A, B, C, D), and the IL-6R molecule was fitted onto the GHR structure, which is bound to site I of GH.[6] The next steps were performed in an iterative manner. First, the interaction areas were inspected for overlapping side chains. These unfavorable contacts were then eliminated by rotating them properly. Second, the accessible surface was calculated for this complex to find cavities in the interaction area. If possible, these cavities were filled by adjustment of side chains from the neighborhood. These complexes were then energy-minimized and again analyzed for unfavorable contacts and cavities in the interaction area. This procedure was repeated until a low-energy conformation of the complex was reached. The same procedure was used to accommodate gp130 to site II of GH.[6] A salt bridge was assumed when the distance between

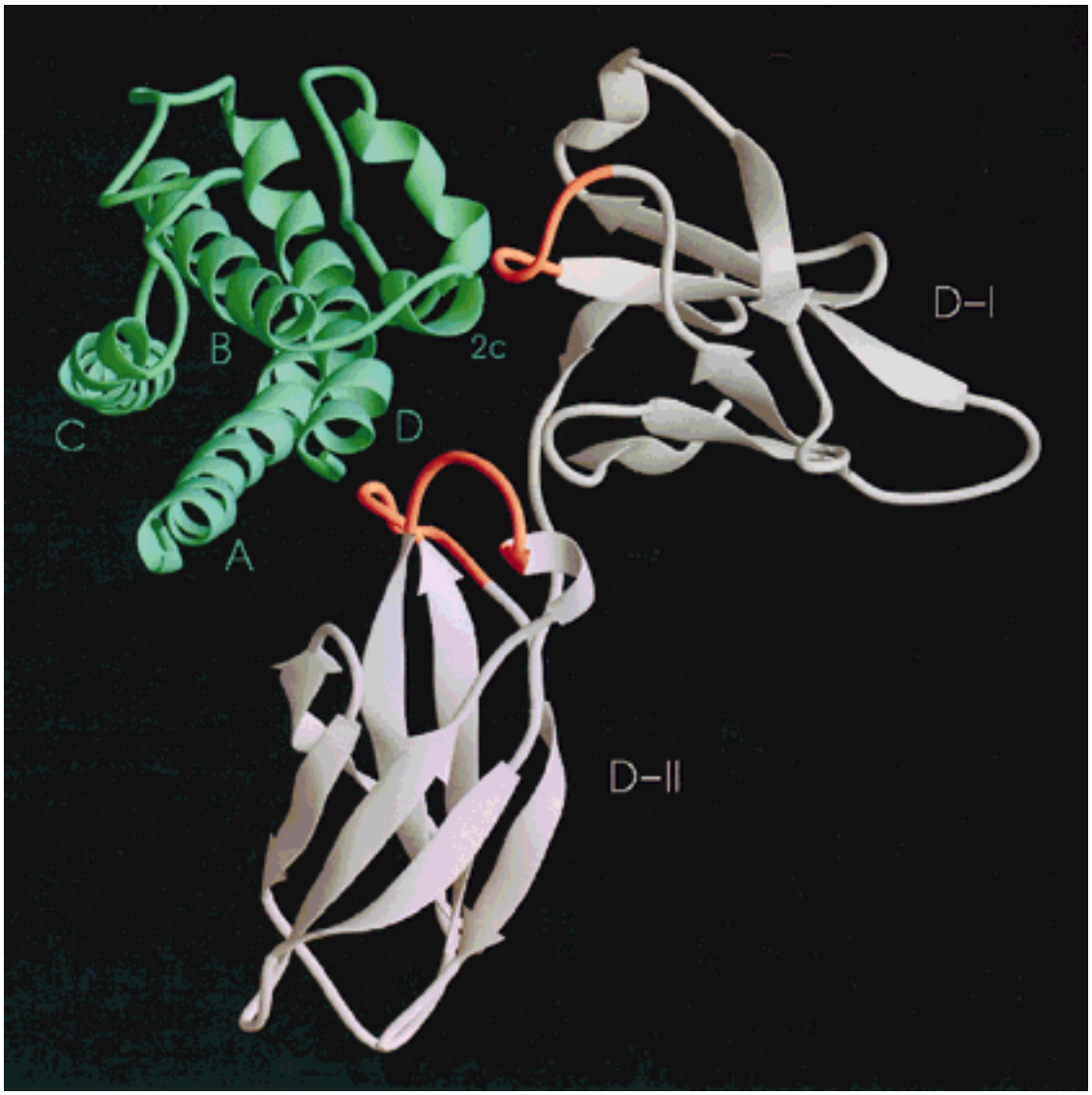

Fig. 1. Ribbon representation of the human IL-6/IL-6R complex. The helices in the IL-6 molecule (green) are designated A, B, C, D. DI (gray) and DII (light violet) designate the two domains of IL-6R. The loops of IL-6R involved in IL-6 binding are red. These are EF in DI and B′C′ and F′G′ in DII.

the centers of charged groups was less than 0.5 nm. A hydrophobic contact was identified when the distance of involved atoms was in the range of the sum of their van der Waals radii.

The structural representation was performed with the RIBBON program.[42] All programs were run on a Silicon Graphics Indigo^2 work station.

RESULTS

The Human IL-6/IL-6R Complex

The hIL-6/hIL-6R complex was built as described under Materials and Methods. Figure 1 shows a ribbon representation of the IL-6/IL-6R model complex. Regions of IL-6 (site I) that are in contact with IL-6R in the complex are the beginning of helix A, the end of helix D, and the C-terminal part of the AB loop, which is called the 2c region.[11] Regions of IL-6R that are in contact with IL-6 are the EF loop in the first domain and the B′C′ and F′G′ loops in the second domain.

The site-directed mutagenesis data available to data are compiled in Table I. These mutagenesis data show that residues 27–30, located in helix A, especially Arg30, are involved in receptor binding. Residues in the 2c region, which in our IL-6 model is located at the end of the AB loop, are also involved, especially the two phenylalanines, 74 and 78. The third region involved is located at the end of helix D

TABLE I. Mutations in IL-6 and IL-6R That Affect the Interaction of the Two Molecules*

	Mutation	Location	Ref.	Effect
IL-6	Δ 1–26	Helix A	22	+++a
	Δ 1–28	Helix A	22	++a
	Δ 1–30	Helix A	22	+a
	R30A	Helix A	51	−b
	R30T, Y31H, I32V	Helix A	47	−b
	G77–E95 (human) → G77–H95 (murine)	Loop AB	11	−b
	F74E	Loop AB	47	−b
	F78X, X = E,P	Loop AB	24,47	−b
	R168M	Helix D	48	−a
	L174V	Helix D	46	+b
	L174R	Helix D	48	−a
	S177R	Helix D	50	−b
	L178X, X = Q,R	Helix D	50	−b
	L178X, X = D,P	Helix D	31	−a
	R179X, X = P,H,N,S,A,G	Helix D	31	−a
	R179K	Helix D	31	++a
	R179E	Helix D	51	−b
	A180X, X = D,T,L,R	Helix D	45,50	+a,−b
	R182X, X = K,Q,A	Helix D	30	++a
	R182X, X = E,T,P	Helix D	30	+a
	R182X, X = G,L,W	Helix D	50	+b
	H164–R182 (human) → Q164–R182 (murine)	Helix D	55	−b
	H164–R182 (human) → Q164–R182 (rat)	Helix D	55	++b
	Δ 182–184	Helix D	28	−a
	Δ 183–184	Helix D	28	++a
IL-6R	Y188X, X = A,G,R,V	Loop EF	35	++b
	S247A, F248V	Loop B′C′	34	−b
	F248X, X = S,V	Loop B′C′	35	−b
	Y249X, X = A,I,V,R	Loop B′C′	35	−b
	R250S, L251I	Loop B′C′	34	−b
	E254A	Loop B′C′	34	++b
	E296R	Loop F′G′	47	−b
	E297R	Loop F′G′	47	−b
	E297A, F298I	Loop F′G′	34	−b
	G301V, E302D	Loop F′G′	34	−b

*+++a, active; ++a, reduced activity; +a, strongly reduced activity (<10%); −a, no activity; ++b, reduced binding; +b, strongly reduced binding (<10%); −b, no binding; Δ, deletion.

in our IL-6 model and contains the three arginines, 168, 179, and 182. Point mutations of each of these residues in IL-6 led to the loss, or at least a reduction, of the binding affinity to IL-6R (see Table I).

Table I also contains data on mutations of the IL-6R molecule that affect binding to IL-6. The mutated positions are located in the EF loop (Tyr188), B′C′ loop (Phe248, Tyr249, Arg250, and Glu254) and F′G′ loop (Glu296, Glu297, Glu302). These residues are all part of the contact region in our model of the complex.

Further analysis of the model revealed that, as in the GH/GHR complex, residues participating in the interaction can be divided into two types: hydrophobic residues, which contribute to the binding energy, and hydrophilic residues, which are responsible for recognition and interaction specificity.[43,44]

Inspection of the distribution and orientation of the charged residues in the interaction area revealed several clusters of salt bridges to which both molecules contribute. The residues of IL-6 and IL-6R forming intermolecular ion pairs according to our model are shown in Figure 2A. In IL-6 these residues are Glu23, Asp26, Lys27, and Arg30 in helix A and Arg168, Arg179, and Arg182 in helix D. From site-directed mutagenesis experiments it is known that most of these residues are indeed important for IL-6/IL-6R complex formation (see Table I). While Arg30, Arg168, Arg179, and Arg182 are essential for receptor binding, Glu23, Asp26, and Lys27 seem to play a minor role in receptor recognition.

In IL-6R the residues involved in these salt-bridge clusters are Arg250, Arg252, Asp272, Glu254, Glu296, Glu297, and Glu302. Exchange of residues Arg250, Glu254, Glu296, Glu297, and Glu302 each results in reduced or no binding of the ligand (see Table I), but

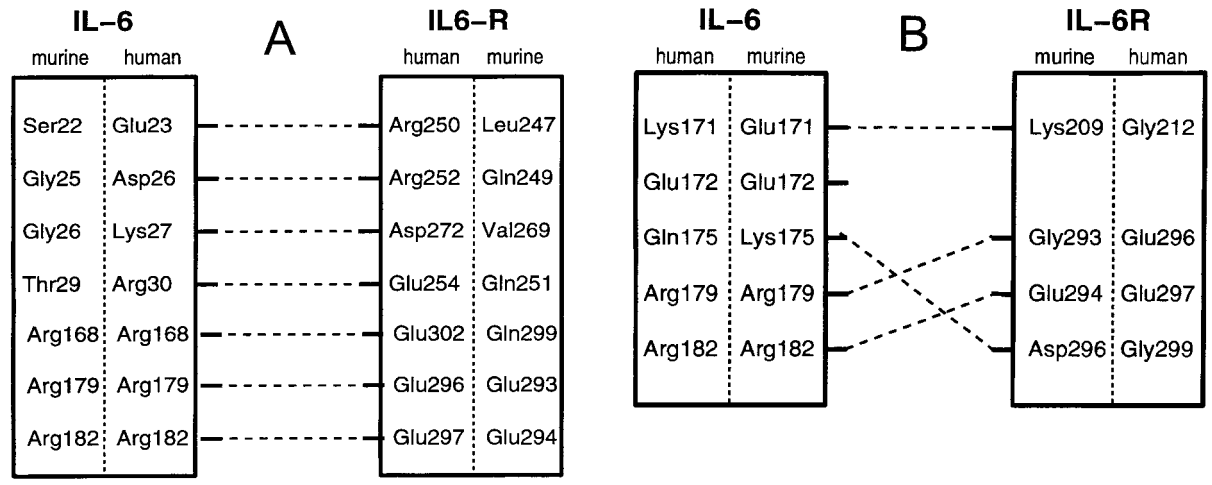

Fig. 2. Schematic representation of the charged residues involved in the interaction of IL-6 and IL-6R. **A:** The charged residues interacting in the human complex and their corresponding residues in the murine complex. **B:** The charged residues interacting in the murine complex and their corresponding residues in the human complex.

Arg252 has not been tested by mutagensis experiments so far. Exchange of Asp272 against Tyr has not shown any effect on IL-6 binding.

In summary: Of the 14 charged residues found in the interface of the complex, 8 are crucial for IL-6 binding, and 6 are of minor or no importance. Figure 3 shows the spatial arrangement of these residues in the contact region of the hIL-6/hIL-6R model complex.

Two distinct regions in the IL-6/IL-6R interface can be defined as hydrophobic contacts:

1. The Phe298 side chain of the receptor is poking into a hydrophobic cleft of the IL-6 molecule formed by residues Ile29, Ile32, Leu33 in helix A and Leu174, Leu178 in helix D. Experimental results have shown that Phe298 in the receptor is essential for the formation of the IL-6/IL-6R complex[33] (see Table I). IL-6 residues Leu174 and Leu178 of helix D (see Table I) have been shown to be involved in receptor binding.[45,46]

2. The second hydrophobic contact in the IL-6/IL-6R complex is an aromatic cluster made up of Phe74 and Phe78 of IL-6 and Phe248 and Tyr249 of IL-6R. All of these residues have been shown by site-directed mutagenesis to be crucial for complex formation.[24,34,35,47] (see Table I).

Figure 4 shows the spatial arrangement of these residues in our model of the hIL-6/hIL-6R complex. The hydrophobic contact comprising Ile29, Ile32, Leu33, Leu174, and Leu178 in IL-6 and Phe298 in IL-6R is surrounded by four salt bridges, namely, Arg179/Glu296, Arg182/Glu297, Arg168/Glu302, and Arg30/Glu254. These salt bridges are shielding the hydrophobic area from the water and are therefore essential for complex formation, as also suggested by the mutagenesis data (see Table I).

The Murine IL-6/IL-6R Complex

The mIL-6/mIL-6R complex was built in the same way as the human complex. The interactions between the two molecules were again analyzed according to the criteria applied there, that is, by dividing them into two categories: interactions between charged side chains (salt bridges) and hydrophobic interactions. Figure 2B shows the pattern of charged interactions in the model of the mIL-6/mIL-6R complex. Although, as in the human complex, these residues are part of larger salt bridge clusters, they were grouped into pairs for clarity. Note that residue 172 (Fig. 2B) in both human and mouse IL-6 is Glu, but it is not involved in the salt bridge cluster in either receptor complex.

As in the human complex, there are two distinct hydrophobic interaction spheres. The first is a hydrophobic pocket in mIL-6 made up of Ile28, Val31, Leu32, Ile35 (helix A) and Leu174, Leu178 (helix D) into which Leu292 of mIL-6R is poking. The second, as in the human complex, is a cluster of the aromatic side chains of Tyr74 and Tyr78 (mIL-6) as well as Tyr250 and Tyr251 (mIL-6R). The central hydrophobic contact, comprising Ile28, Val31, Leu32, Ile35, Leu174, Leu178 of murine IL-6 and Leu292 of mIL-6R, is shielded from the water by four salt bridges, namely, Arg179/Glu293, Arg182/Glu294, Lys175/Asp296, and Glu171/Lys292.

Comparison of the Human and Murine IL-6/IL-6R Complexes

The mIL-6/mIL-6R complex was analyzed with respect to the distribution of charged residues in the contact region, as was the human complex. Figure 2A,B shows a comparison of charged residues involved in the human and murine complexes, respec-

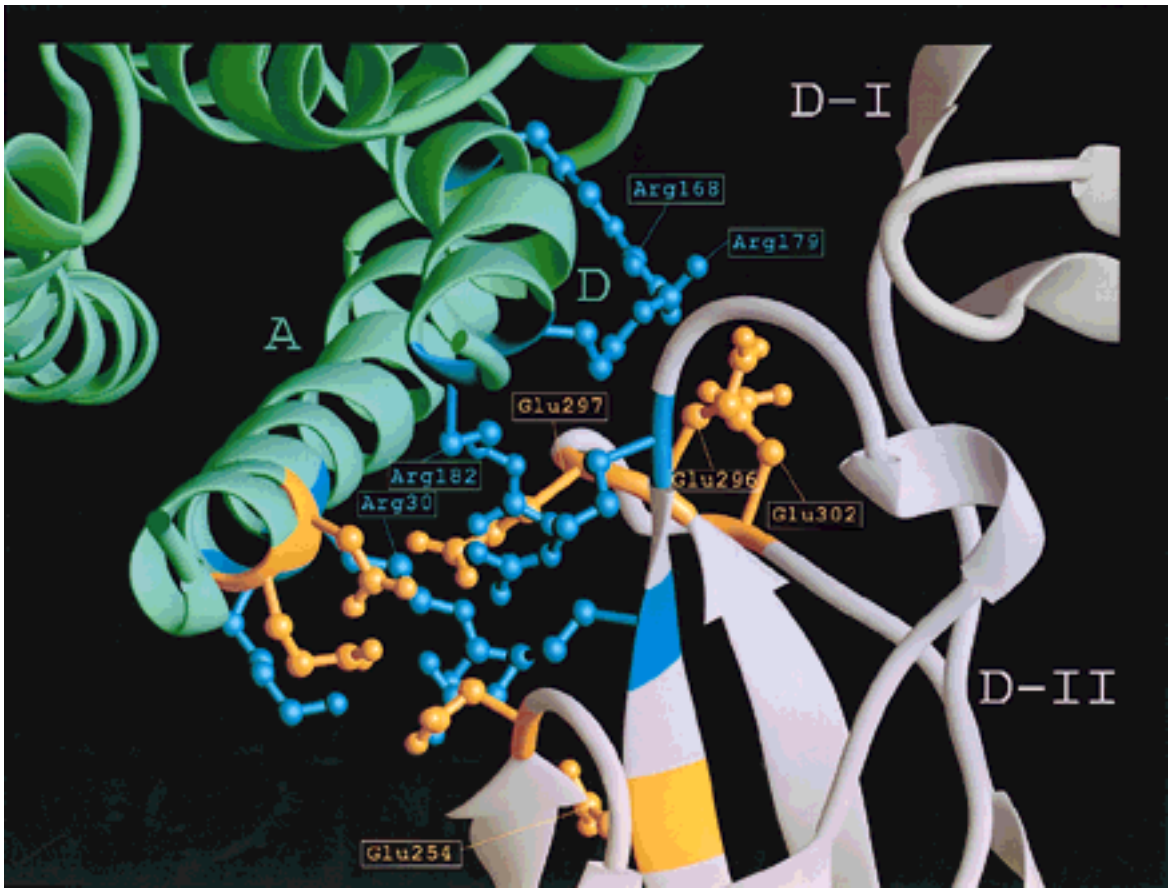

Fig. 3. Ribbon representation of the human IL-6/IL-6R complex. (For color coding see legend of Fig. 1.) Only charged side chains involved in the interaction are depicted. Positively charged side chains are blue, negatively charged side chains are orange.

tively. Some of the salt bridges found in the human complex cannot be formed in the murine complex. Most of the positions in both IL-6 and IL-6R, which contain interacting charged residues in the human system, are held by uncharged residues in the murine system. Conversely, charged residues in the murine system are replaced by uncharged ones in the human system. Furthermore, residue 171 in hIL-6 is Lys, while in the murine system it is Glu. Only two ion pairs are common to both complexes: Arg179/Glu296, Arg182/Glu297, and Arg179/Glu293, Arg182/Glu294 in the human and murine systems, respectively. These pairs of residues are conserved in all species.

Figure 5 shows the alignments of the IL-6 and IL-6R sequences, respectively, from different species. Residues that participate in the interaction are color-coded. Although human and murine complexes differ significantly in the position, nature, and number of charged residues involved in salt-bridge clusters, the hydrophobic residues responsible for the binding energy of the two molecules—or at least their hydrophobic nature—are conserved. According to the number, distribution, and nature of charged residues involved in receptor recognition, the IL-6 sequences can be divided into two subgroups: (1) mouse, rat; (2) man, dog, pig, cow, sheep, and sus scrofa. The different patterns underlying these two groups thus provide an explanation for the species specificity of IL-6/IL-6R complex formation.

Figure 6A shows a comparison of the residues involved in the interaction within the interspecies IL-6/IL-6R complexes. The Lys175 Asp296 ion pair (see also Fig. 2B) in the m/m complex is substituted by the Lys171 Asp296 ion pair in the h/m complex. In the m/h complex this pair is not possible because Asp296 is changed into a Gly. While Glu172 (m- and hIL-6) and Lys202 (mIL-6R) are not forming in a salt bridge in both the h/h (see Fig. 2A) and m/m (see Fig. 2B) complexes, they can form an ion pair in the h/m chimera, which is a substitute for the Glu171 Lys209 pair in the m/m complex. These alternative salt bridges cannot be formed in the IL-6/mIL-6R complex because the mIL-6R residues involved are exchanged against noncharged ones. Figure 6B shows

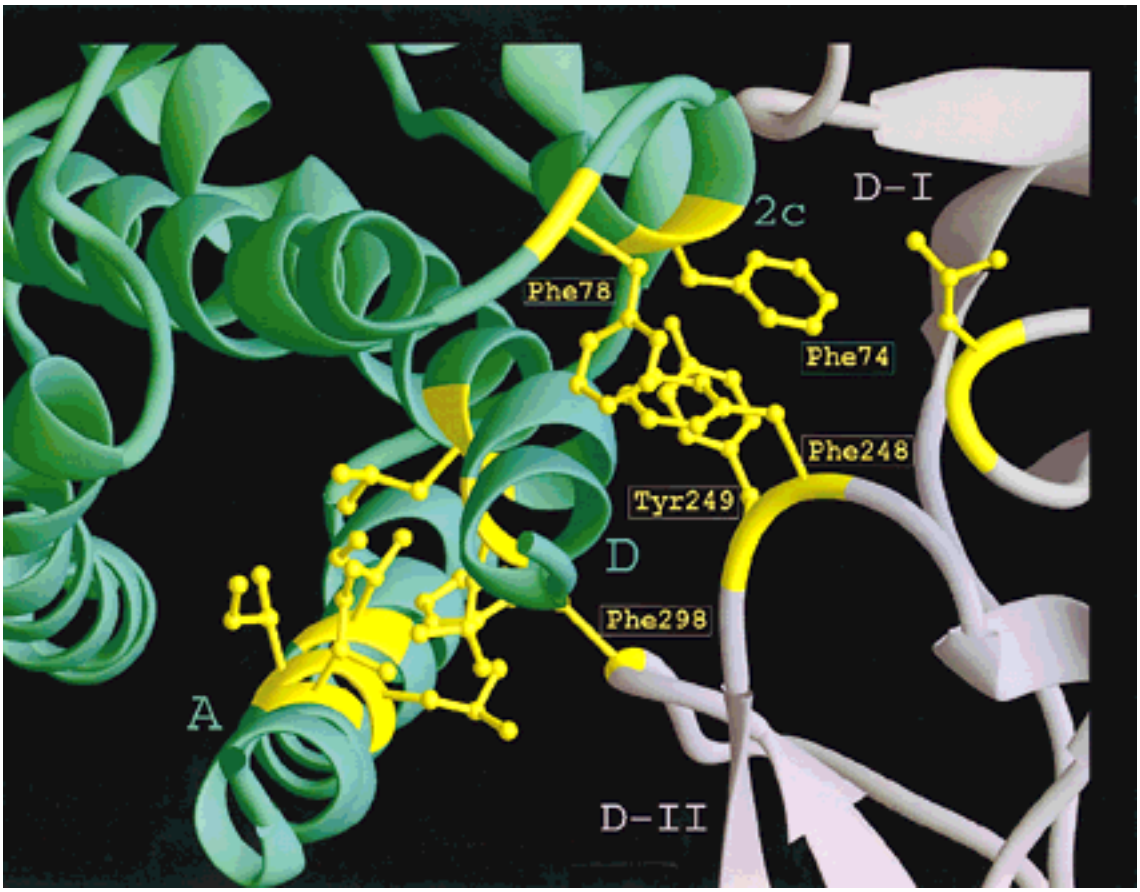

Fig. 4. Ribbon representation of the human IL-6/IL-6R complex. The hydrophobic residues involved in the interaction are depicted in yellow. IL-6 is green, with A and D designating helices and 2c the region containing the two phenylalanines 74 and 78. Domains DI and DII of IL-6R are gray and light violet, respectively.

a schematic drawing of the salt bridge arrangements for the intra- and interspecies complexes.

As in the GH/GHR complex, ion pairs surround the central hydrophobic contact (involving Phe298 and Leu295 in h- and mIL-6R, respectively), and can be formed in hIL-6/hIL-6R, mIL-6/mIL-6R, hIL-6/mIL-6R, but not in mIL-6/hIL-6R. In summary, in the hIL-6/mIL-6R complex alternative ion pairs (compared with the intraspecies ones) can be formed, but in the mIL-6/hIL-6R complex these alternatives do not exist.

The absence of these clusters is supposed to leave the hydrophobic sphere unshielded from water and hence to make it less stable. These predictions were confirmed by the effects of substitutions of some of those amino-acid residues in IL-6 on the capacity to stimulate the growth of the IL-6-dependent murine hybridoma 7TD1 cells.[48,49] Changing Glu172 in IL-6 into Asp decreases binding to the mIL-6R by about 50%[48]; changing it into Val almost maintains wild-type activity on human cells but reduces the activity on mouse cells to only 17%.[50] A double mutant of Lys171Asn and Gln175Leu in hIL-6 abolishes binding to the mIL-6R completely.[48] These results suggest that the alternative salt bridges in the h/m complex exist and support our interpretation of the species specificity of these complexes.

The Human IL-6/IL-6R/gp130 Complex

The cytokine domain that binds one of the two gp130 molecules needed for signal transduction apparently corresponds to site II of GH in the GH/$(GHR)_2$ complex (see Figs. 7 and 8). Experimental support for this comes from site-directed mutagenesis experiments[13,14,20] (see Table II). Based on the above assumption, mutations in helices A and C[13,14] of IL-6 were used to construct IL-6 antagonists that can still bind to IL-6R but inhibit the formation of the signal-transducing complex with gp130. A single-point mutation was insufficient to achieve this. Only four replacements in combination (see Table II, site II) led to an efficient IL-6 antagonist.[14] However, the observed tolerance of site II toward point mutations may be prerequisite for gp130 to interact with different cytokines.

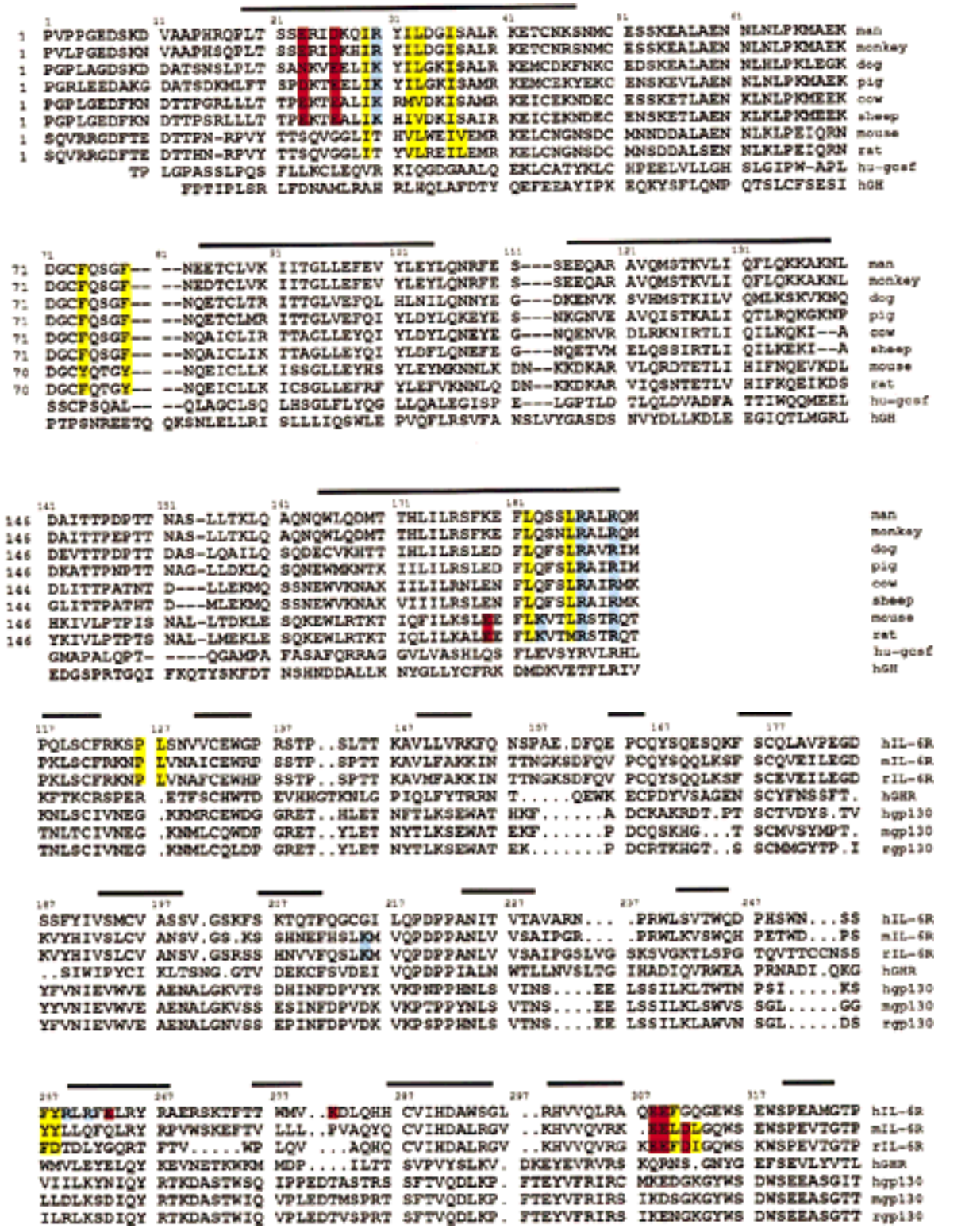

Fig. 5. **A:** Alignment of IL-6 sequences from different species with the GH sequence and G-CSF used as the template for the IL-6 model. **B:** Alignment of IL-6R and gp130 sequences from different species with the GHR used as the template for the IL-6R and gp130 model. Color bars designate residues that are involved in the interaction within the corresponding complexes (yellow, hydrophobic residues; blue, positively charged residues; red, negatively charged residues). Bars above the sequences indicate α helices in the IL-6 (Fig. A) and β sheets in the IL-6R and gp130 (Fig. B) models, respectively.

A

IL–6 *murine*	IL–6R *human*	IL–6 *human*	IL–6R *murine*
Glu171	Gly212	Lys171	Lys209
Glu172		Glu172	
Lys175	Gly299	Gln175	Asp296

B

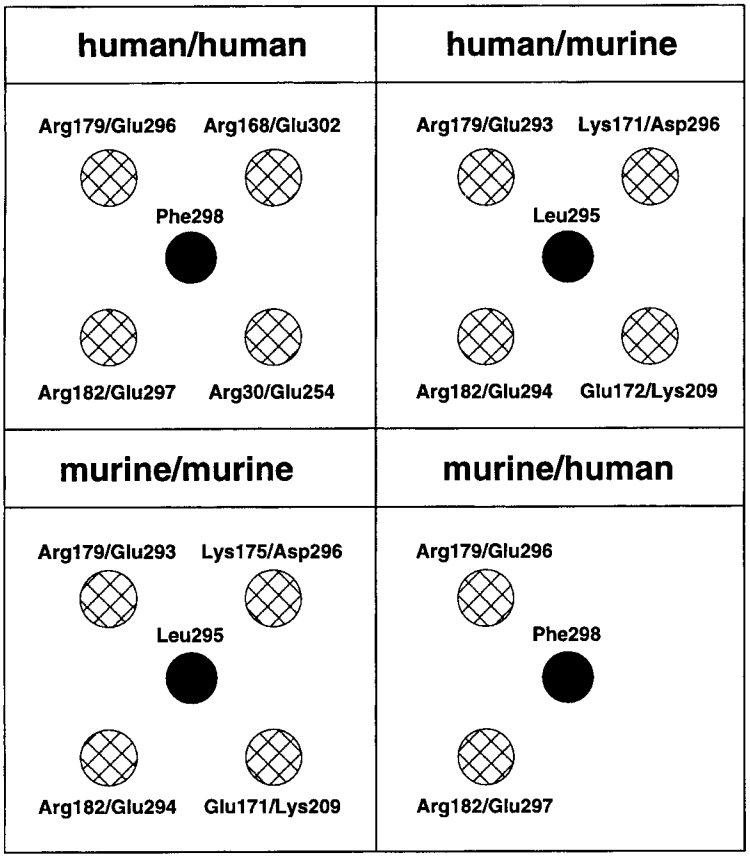

Fig. 6. **A:** Comparison of charged residues involved in salt-bridge clusters in the contact region of the two interspecies IL-6/IL-6R complexes. Arrows designate salt bridges that can form. **B:** Schematic representation of the IL-6/IL-6R contact regions in all possible intra- and interspecies complexes. Black circles represent the central hydrophobic residues of the corresponding receptors that interact with hydrophobic resdidues of the ligand. Crosshatched circles designate pairs of IL-6/IL-6R amino acids engaged in salt bridges. The central hydrophobic interaction is surrounded by four salt bridges in all complexes except in the mIL-6/mIL-6R, where two are lacking.

The analogy to the GH/$(GHR)_2$ complex was also verified by mutations in the contact region of IL-6R with the gp130 molecule. Mutations of IL-6R in this proposed region were able to inhibit the formation of the signal-transducing complex with gp130.[49]

In the IL-6/IL-6R complex there are two hydrophobic interaction spheres surrounded by salt-bridge clusters but only one in the IL-6/gp130 complex. Residues involved are Val121 of IL-6 and Val252 of gp130. Val121 has been confirmed by mutagenesis

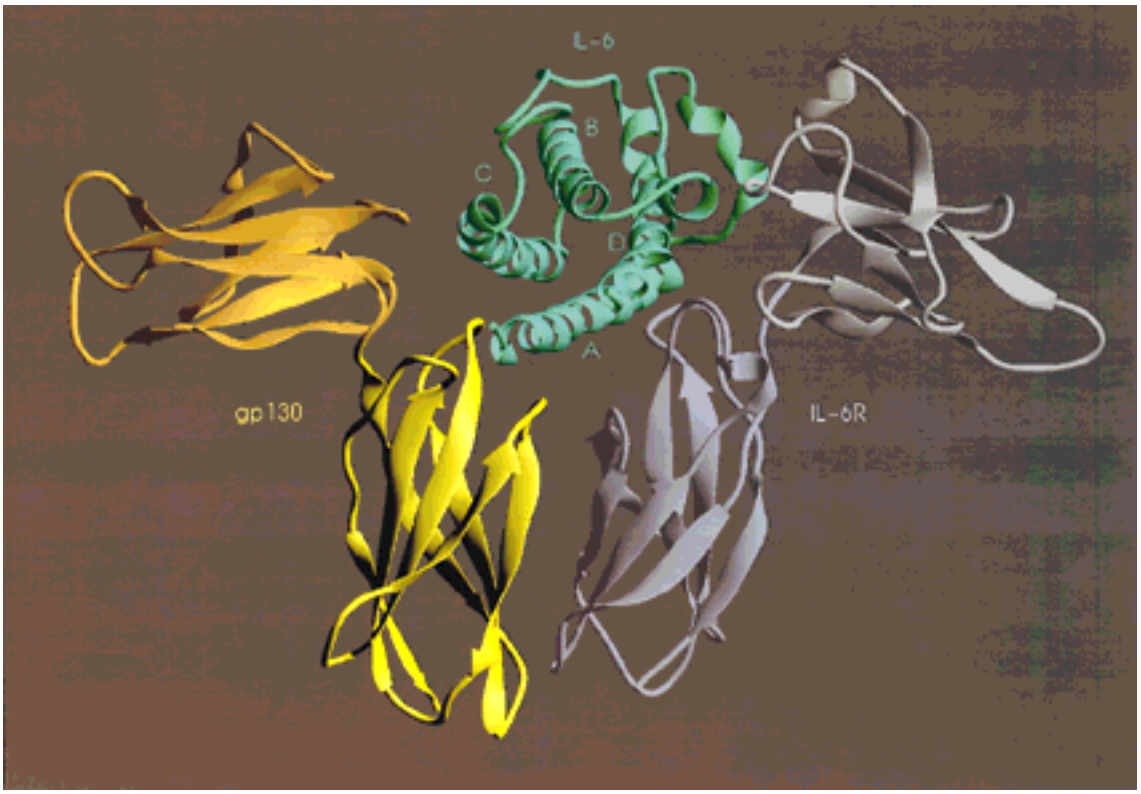

Fig. 7. Ribbon representation of the IL-6/IL-6R/gp130 ternary complex (front view).

(see Table II, site II), but so far no experimental data are available for gp130. However, the model displays the hydrophobic sphere surrounded by a salt-bridge cluster characteristic of a protein complex.

DISCUSSION

Human IL-6/IL-6R Complex

Analysis of our model in view of the mutagenesis data in the literature revealed that most of the residues involved in the interaction area are described to be crucial for the interaction of the two molecules. In the case of IL-6 these are Arg30,[51] Arg168,[48] Arg179,[13,48,50] Arg182,[30,48] Leu174,[46] Leu178,[45] and the 2c region in the AB loop[11] (residues 77–95) (see Fig. 1 and Table I). Our model suggested that the two phenylalanines, 74 and 78, located in the 2c region (see Fig. 4), are the residues important for binding. Mutagenesis results[24,34] (see Table I) have shown that these residues are indeed involved in binding as predicted.

Residues in the IL-6R molecule identified from our model to participate in the interaction are Tyr249, Arg250, Arg252, Glu254, Glu296, Glu297, Phe298, and Glu302. Residues Arg250, Glu254, and Phe248, Tyr249, Phe298, and Glu302 are described in the literature (see Table I) to participate in the complex formation, but not residues Arg250, Glu296, and Glu297. The prediction that residues Glu296 and Glu297 are part of the contact region was again verified by site-directed mutagenesis experiments, which showed that replacing these residues by arginines abolishes IL-6 binding completely.[47]

The random mutagenesis study of Yawata and co-workers[34] showed that ligand binding is disturbed by the substitution of still other residues beyond those listed in Table I. These residues, for instance, the conserved cysteines in domain I and the WSXWS box in domain II, were excluded from Table I because they are conserved in the family of cytokine binding domains. It is therefore likely that they are involved in the hydrophobic core and that their mutation abolishes the binding capacity by structural destabilization rather than by loss of important ligand interactions.

Species Specificity

The agreement of our model with the available site-directed mutagenesis data is a good criterion for its quality. The question now is whether this model can also explain the experimental observation that hIL-6 binds to mIL-6R but that mIL-6 does not bind to hIL-6R.[21]

The region in which two proteins interact in the GH/GHR complex is typically made up as follows: a central hydrophobic sphere is surrounded by salt-bridge clusters[43,44] for protection against water. Thus a protein-protein interface resembles a globular protein. As the removal of the outer hydrophilic residues

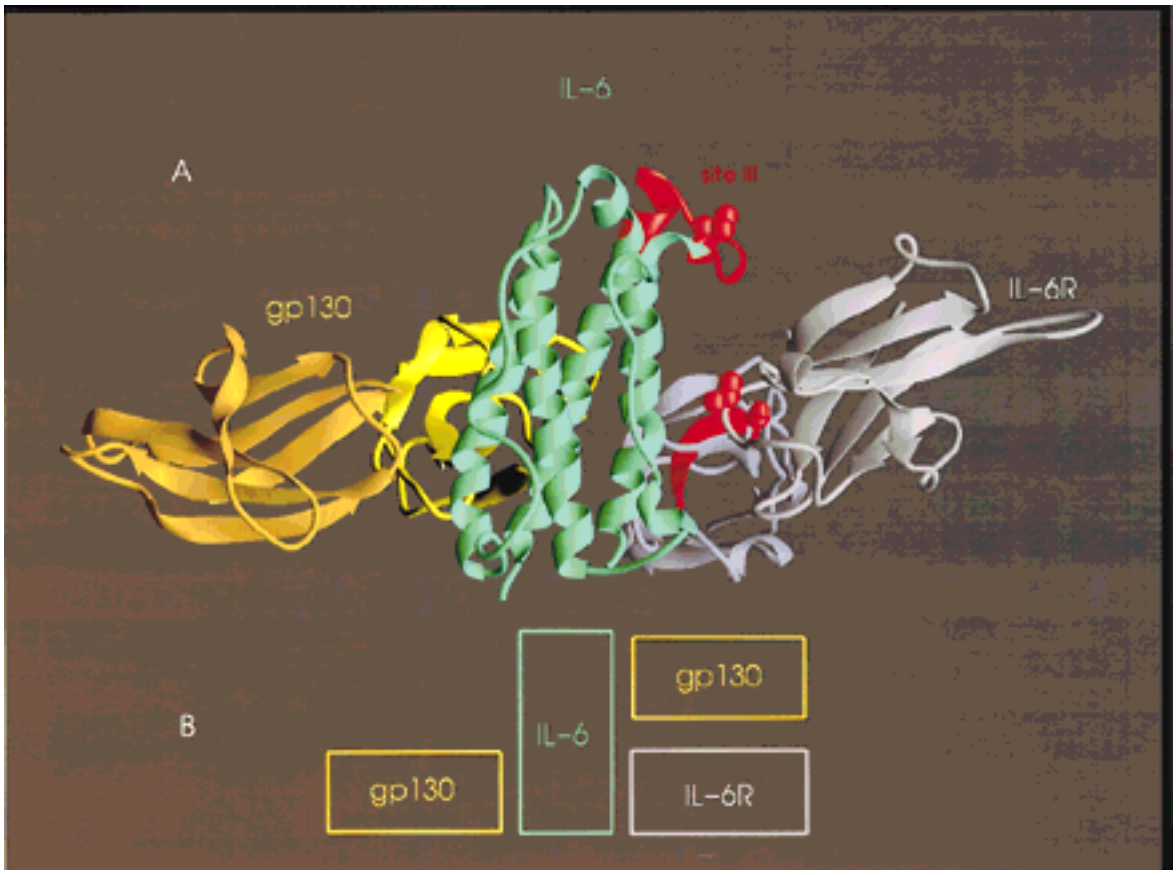

Fig. 8. **A:** Ribbon representation of the IL-6/IL-6R/gp130 ternary complex (top view). Regions involved in the binding of another gp130 molecule are highlighted in red. These include Leu57,[23] Lys54,[24] and region 2a[11] (comprised in site III) of IL-6 and Val 190[23] and Ser186[35] as well as the sequence stretch 249–264[24] of IL-6R. **B:** Schematic drawing of the tetrameric complex (top view) showing the possible location of the second gp130 molecule.

from a globular protein would entail denaturation, its consequence for a protein-protein interface would be the loss of binding affinity for structural reasons.

Our model shows that in the hIL-6/hIL-6R, in the mIL-6/mIL-6R, and in the hIL-6/mIL-6R complexes the hydrophobic region of interaction is shielded by clusters of salt bridges, but in the case of the mIL-6/mIL-6R complex it would be unprotected against the water, and therefore this complex would not form. As shown in Figure 6B the central hydrophobic residue Phe298 (IL-6R) or Leu295 (mIL-6R) is surrounded by four salt bridges in the complex. This holds for the hIL-6/mIL-6R but not for the mIL-6/hIL-6R complex, where two of them are missing. This would leave the hydrophobic core of the region of interaction open for access of water and therefore destabilize the complex. We propose that this is how the observation that hIL-6 binds to both human and murine cells, but mIL-6 binds only to murine cells, can be explained at the molecular level.

The IL-6/IL-6R/gp130 Complex

In analogy to the GH/$(GHR)_2$ complex a gp130 molecule can be placed at the corresponding position of site II. Experiments with chimeric h/mIL-6 proteins have predicted that helix C of IL-6 contains residues important for activating gp130 but not for binding IL-6R.[32] Experiments with point mutants of IL-6 have shown that this site is involved in contacting gp130 and that it can be used to design IL-6 receptor antagonists.[13,14] These data fit well with our model. Residues that were mutated in the IL-6 molecule (site II) are Tyr31, Gly35, Ser118, and Val121[13,14] (see also Table II). In our model residues Ser118 and Val121 are in the central region of IL-6/gp130 interaction, while Tyr31 lies on its border.

Mutagenesis studies on IL-6R in the region of the gp130/IL-6R interaction site have already shown that the orientation and location of this gp130 molecule is the same as in the GH/$(GHR)_2$ complex.[34,49] Since no mutagenesis data on gp130 are available, a validation of the IL-6/gp130 interaction site is one-sided, as it only relies on data obtained with IL-6 muteins. Our model can now be used to guide site-directed mutations of gp130 for further verification and refinement.

TABLE II. Mutations in IL-6 and IL-6R That Affect the Complex Formation Between IL-6/IL-6R and gp130 Without Disturbing the Binding of IL-6 to IL-6

	Mutation	Location	Ref.	Effect
IL-6 Site III	Q159E, T162P	Helix D	22	−a
	W157R, D160R	Helix D	20	+a
	T162D	Helix D	20	+a
	Q159E, T163P and K41–A56 (human) → (murine)	Helix D and Loop AB	32	−a
	K54X, X = D	Loop AB	24	++a
	K54X, X = E	Loop AB	24	+a
	K54X, X = F,P	Loop AB	24	a
	L57A	Loop AB	23	++a
IL-6 Site II	Y31D, G35F	Helix A	13	+a
	Y31D, G35F, S118R, V121D	Helix A, C	14	−a
IL-6R	V190G	Loop EF	34,47	−a
	H280I	Loop E′F′	34	−a
	H280S, D281V	Loop E′F′	49	−a
	D281G	Loop E′F′	34	−a
	G285D	Loop E′F′	34	−a
	H288Y, V289L	Strand F′	34	−a

*+++a, active; ++a, reduced activity; +a, strongly reduced activity (<10%); −a, no activity.

The IL-6/IL-6R/$(gp130)_2$ Complex

The ternary IL-6/gp80/gp130 complex can be built by using the x-ray structure of GH/$(GHR)_2$ as a template, but the exact position of the second gp130 molecule remains unknown. Ultracentrifugation results of Ward and colleagues[37] and conclusions of Paonessa and colleagues[20] derived from coprecipitation experiments seem to indicate that the complex in solution consists of two IL-6, two IL-6R, and two gp130 molecules. On the other hand, crystallization and mutagenesis data on CNTF have been interpreted in favor of a tetrameric complex of one molecule each of CNTF, CNTF-R, gp130, and LIFR.[8,52] De Serio and coworkers[53] have presented evidence for a hexameric complex consisting of two CNTF, two CNTFR, one gp130, and one LIFR molecules in solution. Such hexameric complexes possibly existing in solution are not necessarily representative of the membrane situation.

In a hexameric model[20] of the functional complex, two IL-6 molecules, each bound to one IL-6R and one gp130, would be connected by a dimerization interface formed by site III (see Table II), which has been shown to be important for the formation of the functional IL-6 receptor complex.[11,12,23,24] The size of the four-helix bundle protein IL-6, however, which is about twice as long as it is wide, would not allow for a contact between the two gp130 proteins involved (Fig. 8A) in such a hexameric complex. This point is of particular importance in the case of those IL-6-like cytokines that require a gp130/LIFR heterodimer for signal transduction. The hexameric model would predict that the same site of CNTF, for example, contacts gp130 and the LIFR. It is hard to imagine how homodimerization of gp130 or heterodimerization of gp130 and LIFR could be achieved specifically without direct contact of the partner molecules.

A possible explanation for the detection of a hexameric complex of IL-6, soluble IL-6R, and soluble gp130 in solution could be that, at the high protein concentrations used, dimerization of the ligand occurred in a similar fashion as it has been observed for CNTF in the crystal and in solution.[8] Since dimerization occurs via helices C and B, each monomer in such an IL-6 dimer should still be able to bind one IL-6R and one gp130 molecule. Hexameric complexes of that kind, however, would not correspond to the functional complexes that are formed at the cell surface and may not be able to transduce a signal.

A tetrameric model of the IL-6 complex would predict that two gp130 molecules are directly bound to IL-6. This notion is supported by mutagenesis studies by Yawata and associates[34] and our group.[11,12,23,24,35] Mutagenesis in regions of IL-6 and IL-6R, highlighted in red in Figure 8A, resulted in muteins that were unchanged in their interaction with the IL-6R, but failed to form a signal-transducing complex, most likely because gp130 binding was impaired. Moreover, a peptide of the IL-6R, which inhibits formation of the signal-transducing complex,[54] is located in the same region (see Fig. 8A). We therefore suggest that the second gp130 molecule does contact those regions and that the IL-6 receptor complex has the stoichiometry shown in Figure 8B. An obvious advantage of this model is that it provides a mechanism for the gp130 homodimerization or gp130/LIFR heterodimerization. In cytokines that require a gp130/LIFR heterodimer the region corresponding to site III in IL-6, which is a gp130 interaction site, would be a binding site for LIFR. This hypothesis can be verified by constructing chimeric molecules consisting of CNTF and IL-6, for example. Implantation of the gp130 interaction site into the

CNTF protein should result in a CNTF molecule that forms a complex with two gp130 molecules.

ACKNOWLEDGMENTS

We thank Gerhard Müller-Newen, Ursula Horsten, Oliver Weiergräber (RWTH Aachen, Germany) and Floris de Hon and Just Brakenhoff (CLB, Amsterdam, the Netherlands) and Marc Ehlers (University of Mainz, Germany) for sharing data before publication and helpful discussions. This work was funded by grants from the Deutsche Forschungs-Gemeinschaft to J.G. and A.W. (He 906/18-1) and to S.R.-J. (Ro 632/7-1).

REFERENCES

1. Miyajima, A.T., Kitamura, T., Harada, N., Yokata, T., Arai, K. Cytokine receptors and signal transduction. Annu. Rev. Immunol. 10:295–331, 1992.
2. Akira, S., Taga, T., Kishimoto, T. Interleukin-6 in biology and medicine. Adv. Immunol. 54:1–72, 1993.
3. Mackiewicz, A., Koi, A., Sehgal, P. Interleukin-6-type cytokines. Ann. N.Y. Acad. Sci. 762:1–509, 1995.
4. Bazan, J.F. Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. U.S.A. 87:6934–6938, 1990.
5. Sprang, S.R., Bazan, J.F. Cytokine structural taxonomy and mechanisms of receptor engagement. Curr. Opin. Struct. Biol. 3:815–827, 1993.
6. De Vos, A.M., Ultsch, M., Kossiakoff, A.A. Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. Science 255:306–312, 1992.
7. Robinson, R.C., Grey, L.M., Staunton, D., Vankelecom, H., Vernallis, A.B., Moreau, J.F., Stuart, D.I., Heath, J.K., Jones, E.Y. The crystal structure and biological function of leukemia inhibitory factor: Implications for receptor binding. Cell 77:1101–1116, 1994.
8. McDonald, N.Q., Panayotatos, N., Hendrickson, W.A. Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing. EMBO J. 14:2689–2699, 1995.
9. Hill, C.P., Osslund, T.D., Eisenberg, D. The structure of granulocyte colony stimulating factor and its relationship to other growth factors. Proc. Natl. Acad. Sci. U.S.A. 90:5167–5171, 1993.
10. Zink, T., Ross, A., Lüers, K., Cieslar, C., Rudolph, R., Holak, T.A. Structure and dynamics of the human granulocyte colony stimulating factor determined by NMR spectroscopy: Loop mobility in a four-helix bundle protein. Biochemistry 33:8453–463, 1994.
11. Ehlers, M., Grötzinger, J., de Hon, F.D., Mullberg, J., Brakenhoff, J.P.J., Liu, J.W., Wollmer, A., Rose-John, S. Identification of two novel regions of hIL-6 responsible for receptor binding and signal transduction. J. Immunol. 153:1744–1753, 1994.
12. Ehlers, M., de Hon, F.D., Bos, K.H., Horsten, U., Kurapkat, G., Schmitz van De Leurs, H., Grötzinger, J., Wollmer, A., Brakenhoff, J.P.J., Rose-John, S. Combining two mutations of human interleukin-6 that affect gp130 activation results in a potent interleukin-6 receptor antagonist on human myeloma cells. J. Biol. Chem. 270:8158–8163, 1995.
13. Savino, R., Lahm, A., Salvati, A.L., Ciapponi, L., Sporeno, E., Altamura, S., Paonessa, G., Toniatti, C., Ciliberto, G. Generation of interleukin-6 receptor antagonists by molecular modeling guided mutagenesis of residues important for gp130 activation. EMBO J. 13:1357–1367, 1994a.
14. Savino, R., Ciapponi, L., Lahm, A., Demartis, A., Cabibbo, A., Toniatti, C., Delmastro, P., Altamura, S., Ciliberto, G. Rational design of a receptor superantagonist of human interleukin-6. EMBO J. 13:5863–5870, 1994b.
15. Krüttgen, A., Grötzinger, J., Kurapkat, G., Weiss, J., Simon, R., Thier, M., Schröder, M., Heinrich, P.C., Wollmer, A., Comeau, M., Müllberg, J., Rose-John, S. Human ciliary neurotrophic factor: A structure-function analysis. Biochem. J. 309:215–220, 1995.
16. Cosman, D., Lyman, S.D., Idzerda, R.L., Beckmann, M.P., Park, L.S., Goodwin, R.G., March, C.J. A new cytokine receptor superfamily. Trends Biochem. Sci. 15:265–270, 1990.
17. Horsten, U., Schmitz van De Leur, H., Müllberg, J., Heinrich, P.C., Rose-John, S. The membrane distal half of gp130 is responsible for the formation of a ternary complex with IL-6 and the IL-6 receptor. FEBS Lett. 360:43–46, 1995.
18. Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T., Kishimoto, T. IL-6 induced homodimerization of gp130. Science 260:1808–1810, 1993.
19. Somers, W., Ultsch, M., De Vos, A.M., Kossiakoff, A.A. The x-ray structure of a growth hormone prolactin receptor complex. Nature 372:478–481, 1994.
20. Paonessa, G., Graziani, R., De Serio, A., Savino, R., Ciapponi, L., Lahm, A., Salvati, A.L., Toniatti, C., Ciliberto, G. Two distinct and independent sites on IL-6 trigger gp130 dimer formation and signalling. EMBO J. 14:1942–1951, 1995.
21. Van Snick, J. Interleukin-6: An overview. Annu. Rev. Immunol. 8:253–279, 1990.
22. Brakenhoff, J.P.J., de Hon, F.D., Fontaine, V., ten Boekel, E., Schooltink, H., Rose-John, S., Heinrich, P.C., Content, J., Aarden, L.A. Development of a human interleukin-6 receptor antagonist. J. Biol. Chem. 269:86–93, 1994.
23. de Hon, Klaasse Bos, H., Ebeling, S.B., Grötzinger, J., Kurapkat, G., Rose-John, S., Aarden, L.A., Brakenhoff, J.P.J. Leucine-58 in the putative 5th helical region of human interleukin (IL)-6 is important for activation of the IL-6 signal transducer, gp130. FEBS Lett. 369:187–191, 1995.
24. Ehlers, M., Grötzinger, J., Fischer, M., Bos, H.K., Brakenhoff, P.J., Rose-John, S. Identification of single amino acid residues of human Interleukin-6 involved in receptor binding and signal initiation. J. Interferon Cytokine Res. 16:569–576, 1996.
25. Brakenhoff, J.P.J., Hart, M., Aarden, L.A. Analysis of hIL-6 mutants expressed in *Escherichia coli:* Biologic activities are not affected by deletion of amino acids 1–28. J. Immunol. 143:1175–1182, 1989.
26. Brakenhoff, J.P.J., Hart, M., de Groot, E.R., Di Padova, F., Aarden, L.A. Structure-function analysis of human IL-6: Epitope mapping of neutralizing monoclonal antibodies with amino and carboxyl terminal deletion mutants. J. Immunol. 145:561–568, 1990.
27. Fontaine, V., Dewit, L., Brakenhoff, J., Content, J. Structure-function studies of human interleukin-6. Mol. Cell. Biol. Cytokines 10:45–50, 1990.
28. Krüttgen, A., Rose-John, S., Dufhues, G., Bender, S., Lütticken, C., Freyer, P., Heinrich, P.C. The three carboxyl terminal amino acids of human interleukin-6 are essential for its biological activity. FEBS Lett. 273:95–98, 1990.
29. Krüttgen, A., Rose-John, S., Möller, C., Wroblowski, B., Wollmer, A., Müllberg, J., Hirano, T., Kishimoto, T., Heinrich, P.C. Structure-function analysis of human interleukin-6: Evidence for the involvement of the carboxy terminus in function. FEBS Lett. 262:323–326, 1990.
30. Lütticken, C., Krüttgen, A., Möller, C., Heinrich, P.C., Rose-John, S. Evidence for the importance of a positive charge and an α-helical structure of the C-terminus for biological activity of human IL-6. FEBS Lett. 282:265–267, 1991.
31. Fontaine, V., Savino, R., Arcone, R., Dewit, L., Brakenhoff, J., Content, J., Ciliberto, G. Involvement of the Arg179 in the active site of human IL-6. Eur. J. Biochem. 211:749–755, 1993.
32. Van Dam, M., Müllberg, J., Schooltink, H., Stoyan, T., Brakenhoff, J.P.G., Graeve, L., Heinrich, P.C., Rose-John, S. Structure-function analysis of interleukin-6 utilizing human/murine chimeric molecules: Involvement of two

separate domains in receptor binding. J. Biol. Chem. 268:15285–15290, 1993.
33. de Hon, F.D., Ehlers, M., Rose-John, S., Ebeling, S., Bos, H.K., Aarden, L.A., Brakenhoff, J.P.J. Development of an interleukin (IL)-6 receptor antagonist that inhibits IL-6 dependent growth of human myeloma cells. J. Exp. Med. 180:2395–2400, 1994.
34. Yawata, H., Yasukawa, K., Natsuka, S., Murakami, M., Yamasaki, K., Hibi, M., Taga, T., Kishimoto, T. Structure-function analysis of human IL-6 receptor: Dissociation of amino acid residues required for IL-6 binding and for IL-6 signal transduction through gp130. EMBO J. 12:1705–1712, 1993.
35. Kalai, M., Montero-Julian, F.A., Grötzinger, J., Wollmer, A., Morelle, D., Brochier, J., Rose-John, S., Heinrich, P.C., Brailly, H., Content, J. Participation of two Ser-Ser-Tyr-Tyr repeats in IL-6 binding sites of human interleukin-6 receptor. Eur. J. Biochem. 238:714–723, 1996.
36. Weiergräber, O., Schneider-Mergener, J., Grötzinger, J., Wollmer, A., Küster, A., Exner, M., Heinrich, P.C. Use of immobilized synthetic peptides for the identification of contact sites between human interleukin-6 and its receptor. FEBS Lett. 379:122–126, 1995.
37. Ward, L.D., Howlett, G.J., Discolo, G., Yasukawa, K., Hammacher, A., Moritz, R.L., Simpson, R.J. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp130. J. Biol. Chem. 269:23286–23289, 1994.
38. Husar program package, EMBL, Heidelberg, Germany.
39. Rost, B., Sander, C. Combining evolutionary information and neural networks to predict protein secondary structure. Proteins 19:55–72, 1994.
40. Vriend, G. WHAT IF: A molecular modeling and drug design program. J. Mol. Graph. 8:52–6, 1990.
41. an Gunsteren, W.F. Distributed by BIOMOS biomolecular software b.v., Laboratory of Physical Chemistry, University of Groningen, The Netherlands).
42. Carson, M. Ribbons 2.0. J. Appl. Crystallogr. 24:946–950, 1991.
43. Clackson, T., Wells, J.A. A hot spot of binding energy in a hormone receptor interface. Science 267:383–386, 1995.
44. Wells, J.A. Structural and functional epitopes in the growth hormone receptor complex. Biotechnology 13:647–651, 1995.
45. Savino, R., Lahm, A., Giogio, M., Cabibbo, A., Tramontano, A., Ciliberto, G. Saturation mutagenesis of the human interleukin-6 receptor binding site: Implications for its three-dimensional structure. Proc. Natl. Acad. Sci. U.S.A. 90:4067–4071, 1993.
46. Nishimura, C., Ekida, T., Nomura, K., Sakamoto, K., Suzuki, H., Yasukawa, K., Kishimoto, T., Arata, Y. Role of leucine residues in the C-terminal region of human interleukin-6 in the biological activity. FEBS Lett. 311:271–275, 1992.
47. Kalai, M., Montero-Julian, F.A., Grötzinger, J., Fontaine, V., Vandenbussche, P., Deschuyteneer, R., Wollmer, A., Brailly, H., Content, J. Analysis of the IL-6/IL-6 binding interface at the amino acid level. Proposed mechanisms of interaction. Blood (in press).
48. Fontaine, V., Ooms, J., Content, J. Mutagenesis of the human interleukin-6 fourth predicted alpha helix: Involvement of the Arg168 in the binding site. Eur. J. Immunol. 24:1041–1045, 1994.
49. Salvati, A.L., Lahm, A., Paonessa, G., Ciliberto, G., Toniatti, C. Interleukin-6 (IL-6) antagonism by soluble IL-6 receptor alpha mutated in the predicted gp130 binding interface. J. Biol. Chem. 270:12242–12249, 1995.
50. Leebeek, F.W.G., Kariya, K., Schwabe, M., Fowlkes, D.M. Identification of a receptor binding site in the carboxyl terminus of human interleukin-6. J. Biol. Chem. 267:14832–14838, 1992.
51. de Hon, F. "Development and Characterization of Human Interleukin-6 Receptor Antagonists." Dissertation. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, University of Amsterdam, The Netherlands, 1995.
52. Panayotatos, N., Radziejewska, E., Acheson, A., Somogyi, R., Thadani, A., Hendrickson, W.A., McDonald, N.Q. Localization of functional receptor epitopes on the structure of ciliary neurotrophic factor indicates a conserved, function-related epitope topography among helical cytokines. J. Biol. Chem. 270:14007–14014, 1995.
53. De Serio, A., Graziani, R., Laufer, R., Ciliberto, G., Paonessa, G. In vitro binding of ciliary neurotrophic factor to its receptors: Evidence for the formation of an IL-6-type hexameric complex. J. Mol. Biol. 254:795–800, 1995.
54. Grube, B.J., Cochrane, C.G. Identification of a regulatory domain of the interleukin-6 receptor. J. Biol. Chem. 269: 20791–20797, 1994.
55. Leebeck, F.W.G., Fowlkes, D.M. Construction and functional analysis of hybrid interleukin-6 variants. FEBS Lett. 306:262–264, 1992.
56. Arcone, R., Fontaine, V., Coto, I., Brakenhoff, J.P.J., Content, J., Ciliberto, G. Internal deletions of amino acids 29–42 of human interleukin-6 (IL-6) differentially affect bioactivity and folding. FEBS Lett. 288:197–200, 1991.
57. Ehlers, M., Grötzinger, J., de Hon, F.D., Müllberg, J., Brakenhoff, J.P.J., Wollmer, A., Rose-John, S. Residues 77–95 of the human interleukin-6 protein are responsible for receptor binding and residues 41–56 for signal transduction. Ann. N.Y. Acad. Sci. 762:400–402, 1995.