The EMBO Journal vol.14 no.9 pp.1942–1951, 1995

Two distinct and independent sites on IL-6 trigger gp130 dimer formation and signalling

Giacomo Paonessa[1], Rita Graziani, Anna De Serio, Rocco Savino, Laura Ciapponi, Armin Lahm, Anna Laura Salvati, Carlo Toniatti and Gennaro Ciliberto

Istituto di Ricerche di Biologia Molecolare (IRBM), P.Angeletti, Via Pontina Km 30 600, I-00040 Pomezia, Rome, Italy

[1]Corresponding author

The helical cytokine interleukin (IL) 6 and its specific binding subunit IL-6Rα form a 1:1 complex which, by promoting homodimerization of the signalling subunit gp130 on the surface of target cells, triggers intracellular responses. We expressed differently tagged forms of gp130 and used them in solution-phase binding assays to show that the soluble extracellular domains of gp130 undergo dimerization in the absence of membranes. *In vitro* receptor assembly reactions were also performed in the presence of two sets of IL-6 variants carrying amino acid substitutions in two distinct areas of the cytokine surface (site 2, comprising exposed residues in the A and C helices, and site 3, in the terminal part of the CD loop). The binding affinity to IL-6Rα of these variants is normal but their biological activity is poor or absent. We demonstrate here that both the site 2 and site 3 IL-6 variants complexed with IL-6Rα bind a single gp130 molecule but are unable to dimerize it, whereas the combined site 2/3 variants lose the ability to interact with gp130. The binding properties of these variants *in vitro*, and the result of using a neutralizing monoclonal antibody directed against site 3, lead to the conclusion that gp130 dimer is formed through direct binding at two independent and differently oriented sites on IL-6. Immunoprecipitation experiments further reveal that the fully assembled receptor complex is composed of two IL-6, two IL-6Rα and two gp130 molecules. We propose here a model representing the IL-6 receptor complex as hexameric, which might be common to other helical cytokines.

Key words: gp130 dimer/IL-6 receptor antagonist/IL-6 receptor complex/IL-6Rα

Introduction

Interleukin (IL) 6 is a multifunctional cytokine that acts on different target cells to induce a variety of biological responses (Van Snick, 1990). Transmembrane glycoproteins that form the IL-6 receptor complex have been identified and cloned (Yamasaki *et al.*, 1988; Hibi *et al.*, 1990). Two membrane proteins are both necessary and sufficient to constitute a functional receptor complex: IL-6Rα (also referred to as the α subunit) and gp130 (also referred to as the β subunit). IL-6Rα specifically binds IL-6 at a relatively low affinity (10^{-9} M; Yamasaki *et al.*, 1988). The IL-6–IL-6Rα complex associates with gp130 at high affinity (10^{-11} M) thus promoting signal transduction inside the cell (Hibi *et al.*, 1990).

Both IL-6Rα and gp130 belong to the haematopoietin receptor superfamily that includes, among others, the growth hormone (GH) receptor (GHbp) (Cosman, 1993). These receptors all share sequence and structural similarities in a 220 amino acid region called the cytokine binding domain (CBD), located in the extracellular portion of the molecule (Bazan, 1990b). Moreover, all the corresponding cytokines are predicted to fold as a bundle of four α-helices (Bazan, 1990a, 1991).

From sequence and structural similarities with the GH receptor complex, whose structure has been solved by X-ray crystallography (de Vos *et al.*, 1992), we built a computer-assisted 3-D model of the IL-6–IL-6Rα–gp130 interaction (Savino *et al.*, 1994a,b; Lahm *et al.*, 1995). In analogy with GH, the IL-6 receptor complex was envisaged as a heterotrimer in which a single IL-6 molecule bridges the IL-6Rα and the gp130 CBDs. In this complex, the IL-6 contact surface with IL-6Rα is formed by residues in the putative D helix and AB loop (site 1), whereas its binding site to gp130 constitutes exposed residues in the A and C helices (site 2; Savino *et al.*, 1994a,b). Furthermore, residues in the E2 strand and AB2 loop of the CBDs of gp130 and IL-6Rα form a third interface (Yawata *et al.*, 1993; Lahm *et al.*, 1995). The reliability of the model was tested by site-directed mutagenesis of both IL-6 and IL-6Rα; variants bearing amino acid substitutions of residues postulated to be at receptor–cytokine or receptor–receptor interfaces confirmed the phenotypes predicted from the model (Savino *et al.*, 1994a,b).

This heterotrimeric model does not, however, explain additional findings. A key event in the IL-6 signalling cascade is the dimerization of the gp130 chain (Murakami *et al.*, 1993) that activates (i) associated tyrosine kinases of the Jak family and (ii) the phosphorylation of a set of intracellular substrates, including the intracytoplasmic domain of gp130 and transcription factors of the STAT family (Stahl *et al.*, 1994; Zhong *et al.*, 1994). These observations have led to the conclusion that a second gp130 molecule participates in the active IL-6 receptor complex.

Additional information on the complexity of the IL-6 receptor complex has emerged from the recently developed IL-6 receptor antagonists. Different strategies have been adopted to design these IL-6 variants, aimed at generating molecules that can still effectively bind IL-6Rα but can carry amino acid substitutions that impair the activation of gp130 by the IL-6–IL-6Rα complex. Interestingly, two strategies developed independently produced IL-6

antagonists with the expected properties by mutating two different portions of the IL-6 four-helix bundle. The first portion is a patch of four exposed residues in the A and C helices, which we called site 2 (Savino *et al.*, 1994a,b) because its location is similar to site 2 in GH. The second is formed by residues located at the beginning of the D helix and AB loop [referred to as site II by Brakenhoff *et al.* (1994) and Ehlers *et al.* (1994)], which we call site 3. The location of site 3 does not fit into the GH paradigm.

To investigate further the unique properties of the IL-6 receptor complex, we developed a very sensitive assay to analyse the biochemical steps of receptor assembly. Differentially tagged versions of soluble gp130 and IL-6Rα receptors are expressed in insect cells and are used in immunoprecipitation reactions with wild-type and variant IL-6 and IL-6Rα molecules. Our results indicate that the IL-6−IL-6Rα complex binds two gp130 molecules at two distinct and independent binding sites. Furthermore, we provide evidence that two IL-6, two IL-6Rα and two gp130 molecules are present in the final complex, and give details on their topological location in this putative 'hexamer'. A model is presented which reconciles this structure with that of the trimeric GH receptor complex and suggests that a similar assembly process might also take place for other helical cytokines.

Results

An in vitro assay for gp130 homodimerization

Homodimerization of gp130 has been shown to take place on the surface of cells exposed to IL-6 (Murakami *et al.*, 1993). To test whether gp130 requires the transmembrane and intracytoplasmic regions to dimerize or, on the contrary, is an intrinsic property of the extracellular domain, we decided to set up an *in vitro* dimerization assay.

To do this, we expressed gp130 in its soluble form (sgp130), truncated at the predicted junction between extracellular and transmembrane domains, and modified at the C-terminus so as to carry two different epitope tags: sgp130-myc carrying a myc tag (Evan *et al.*, 1985) and sgp130-FLAG with a FLAG tag (Hopp *et al.*, 1988). This makes the different sgp130 molecules recognizable by specific monoclonal antibodies. Likewise, two recombinant soluble forms of IL-6Rα were generated, one truncated at residue 322 of the extracellular domain, the other with a myc tag attached to the same C-terminal residue. All cDNAs encoded their natural leader peptide sequence. The modified cDNAs were inserted in the recombination vector under the baculovirus polyhedrin promoter. Recombinant plasmids were transfected in Sf9 cells and the recombinant viruses expressing the various receptors were purified and amplified. Recombinant receptors were produced by infecting insect cells in serum-free medium. Supernatants were assayed by Western blot for recombinant protein production (Figure 1A). Individual receptors were metabolically labelled with [^{35}S]methionine at high specific activity and low background compared with other proteins (Figure 1B). Receptors expressed in insect cells show a molecular weight of ~40 kDa for sIL-6Rα and 72 kDa for gp130, suggesting that these molecules are subjected to post-translation modifications but to a lesser extent than when expressed in mammalian cells (Sporeno *et al.*, 1994). The expression levels of the two

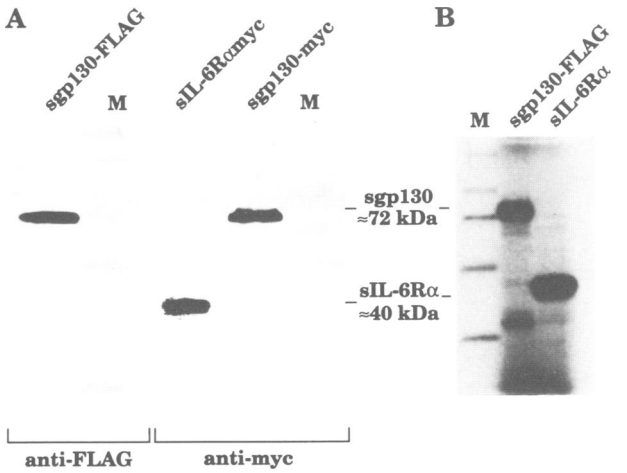

Fig. 1. Production of sgp130 and sIL-6Rα in insect cells. (**A**) Western blotting of supernatants (50 μl) of High Five cells infected with recombinant baculovirus expressing sgp130-FLAG, sIL-6Rαmyc and sgp130-myc and stained with their specific monoclonal antibodies. M, molecular weight marker. (**B**) *In vitro* ^{35}S metabolically labelled sgp130-FLAG and sIL-6Rα (15 μl) expressed in insect cells. M, molecular weight marker.

forms of sgp130 and of sIL-6Rα were estimated to be 1–2 mg/l for sgp130 and 5–7 mg/l for sIL-6Rα after affinity purification (G.Paonessa, unpublished observations).

The strategy adopted for the gp130 dimerization assay is depicted in Figure 2A. In a typical experiment, unlabelled sgp130-myc was immobilized on protein A−Sepharose beads coated with the specific anti-myc monoclonal antibody. After washing, immunoprecipitations were performed in the presence of various combinations of IL-6, IL-6Rα (labelled or unlabelled) and [^{35}S]gp130-FLAG. Because the latter is not recognized by the anti-myc monoclonal antibody, its immunoprecipitation can only be explained by assuming that gp130 dimers had been formed. The results of this experiment are shown in Figure 2B, where the efficient *in vitro* formation of gp130 dimers was confirmed by the immunoprecipitation of labelled gp130-FLAG by anti-myc antibodies. As expected, dimers were only formed in the presence of both IL-6 and IL-6Rα. Likewise, IL-6Rα was immunoprecipitated only in the presence of IL-6 (Figure 2B).

Dimerization of gp130 is triggered by two independent binding sites on IL-6

Recently, IL-6 variants have been generated which act as receptor antagonists (Brakenhoff *et al.*, 1994; Savino *et al.*, 1994a,b). In these variants, amino acid substitutions were introduced in two distinct areas of the IL-6 surface, as predicted by the 3-D model shown in Figure 3. These mutant proteins can be subdivided into two classes: (i) site 2 variants, bearing mutations in helix A and C, and (ii) site 3 variants, bearing mutations at the beginning of helix D. The identification of two distant sites on IL-6 with similar roles in promoting signal transduction, together with the evidence that the signalling subunit undergoes dimerization, immediately suggest that these sites are independent binding sites for the two gp130 subunits.

To test this hypothesis we decided to evaluate their ability to bind baculovirus sgp130 and dimerize it *in vitro*.

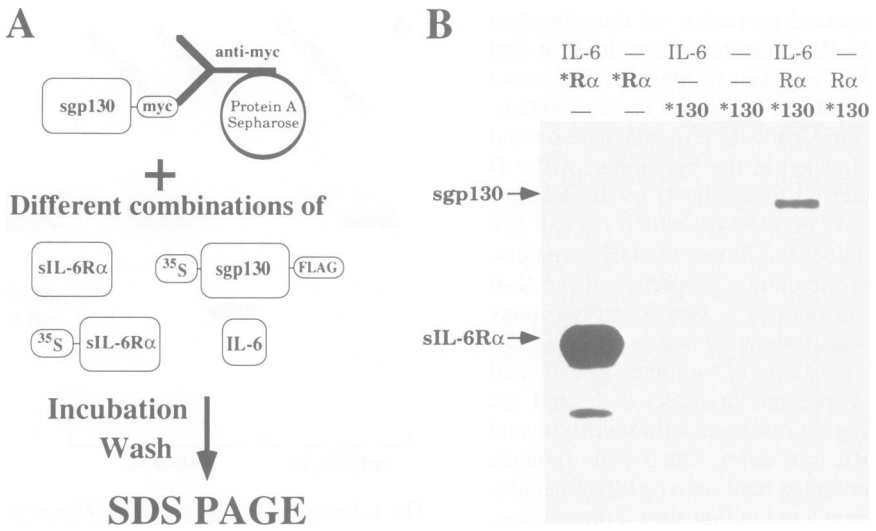

Fig. 2. sgp130 is able to dimerize *in vitro* in the presence of IL-6–sIL-6Rα. (**A**) Experimental strategy. sgp130-myc is attached to protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with different combinations of IL-6, sIL-6Rα and sgp130-FLAG. The participation of a specific molecule in the complex is detected by the use of a ^{35}S-labelled form of a specific receptor chain. After incubation and washing of the Sepharose beads, the bound material is eluted with SDS loading buffer and subjected to PAGE. (**B**) The experiment has been performed according to the experimental strategy shown in (A). Cold sgp130-myc attached on protein A–Sepharose beads was incubated with the molecules indicated on top of each lane in the following amounts: 3 μg IL-6, 500 μl supernatant containing cold sIL-6Rα and 50 μl supernatant containing ^{35}S-labelled receptors. Rα, sIL-6Rα; 130, sgp130-FLAG. Asterisks indicate ^{35}S-labelled material.

From the site 2 variants we selected the four amino acid substitution Y31D/G35F/S118R/V121D, also called DFRD, which acts as a full IL-6 antagonist on a variety of human cell lines (Savino *et al.*, 1994b). For site 3, the variant Q159E/T162P was described which also has low biological activity and acts as a partial antagonist on some but not all human cell lines (Brakenhoff *et al.*, 1994). To avoid introducing proline residues which might affect folding or stability, we generated two new variants (W157R/D160R and T162D) which map in the same area and have similar properties to Q159E/T162P. The properties of all the IL-6 variants used in this study are summarized in Table I, which reports *in vitro* binding to sIL-6Rα as measured by ELISA (Savino *et al.*, 1994a) and biological activity on two different human cell lines with different degrees of sensitivity to human IL-6. Both site 2 and site 3 variants have normal binding affinity to IL-6Rα, as well as a strongly impaired biological activity.

To measure binding to gp130, Sepharose beads coated with anti-myc monoclonal antibody were incubated with the myc-tagged preparation of sIL-6Rα and then used to co-immunoprecipitate labelled sgp130-FLAG in the presence of the wild-type or mutant IL-6 molecule. All three variants (DFRD, W157R/D160R and T162D) immunoprecipitated sgp130 with an efficiency only ~2- to 4-fold lower than wild-type IL-6 (Figure 4A). This result differs from that reported previously with a different site 2 variant 31D/35F using Chinese hamster ovary (CHO)-derived sgp130, where a stronger defect in gp130 binding was observed (Savino *et al.*, 1994a). This discrepancy has also been observed consistently with the site 2 and site 3 variants used in this study (results not shown). We believe this is due to a significant difference in receptor concentration, whereby the use of baculovirus sgp130 reveals residual binding of these variants that cannot be detected under the limiting conditions imposed by the use of the CHO-derived sgp130.

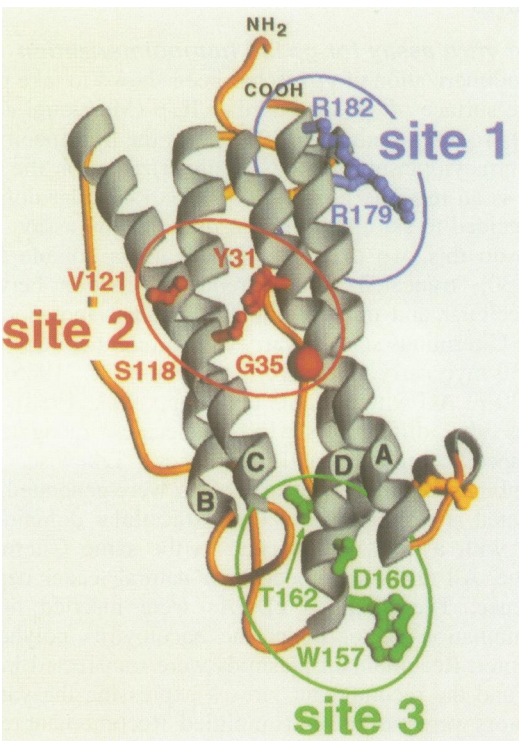

Fig. 3. Location of putative receptor binding sites on the 3-D model of human IL-6. Schematic RIBBONS (Carson, 1987) representation of the IL-6 model (Savino *et al.*, 1994a) showing the three sites involved in receptor interaction: site 1 (blue), the site of interaction with IL-6Rα around Arg179 on helix D (also including parts of the AB loop; Savino *et al.*, 1993); site 2 (red), residues from helix A and C, postulated to be the site of interaction with one gp130 molecule and required for signalling (Savino *et al.*, 1994a,b); site 3 (green), centred around the N-terminal end of helix D, important for signalling and also originally identified as responsible for gp130 binding (Brakenhoff *et al.*, 1994). For site 3, only the IL-6 residues mutagenized in this study are shown.

Table I. Receptor binding and biological activity of the IL-6 variants described in the text

IL-6 variants	sIL-6Rα binding (% of wild-type)	Biological activity (% of wild-type)	
		XG-1 (myeloma cells)	HepG2 (hepatoma cells)
wt IL-6	100	100	100
DFRD	97 ± 15	<0.01	<0.01
157R/160R	220 ± 51	6 ± 4	2.9 ± 0.2
162D	104 ± 18	8 ± 3	1.3 ± 0.3
DFRD/157R/160R	160 ± 40	<0.01	<0.01
DFRD/162D	45 ± 7	<0.01	<0.01

Binding to the sIL-6Rα was determined as described (Savino *et al.*, 1994a). The IL-6 biological activity measured on the XG-1 myeloma cell line is the stimulation of cell replication (Savino *et al.*, 1994b). The IL-6 biological activity measured on the HepG2 hepatoma cell line is the activation of the transfected IL-6-inducible C-reactive protein gene promoter (Savino *et al.*, 1994b). The biological activity of each variant (expressed as percentage of wild-type IL-6 activity) on both cell lines was determined as the ratio between the concentration of wild-type IL-6 and the concentration of variant IL-6 necessary to give 50% of wild-type IL-6 maximal stimulation.

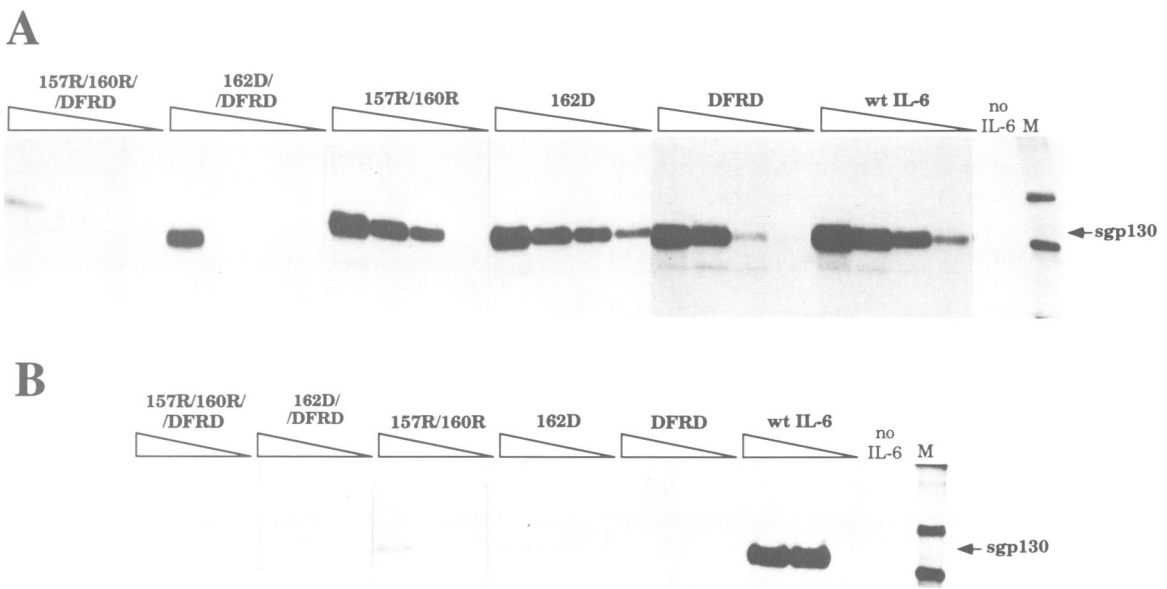

Fig. 4. gp130 binding and dimerization of wild-type and IL-6 variants. (**A**) Binding of sgp130 to wild-type and variants of IL-6 complexed with sIL-6Rα. sIL-6Rαmyc was immobilized on protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with ^{35}S-labelled sgp130-FLAG (50 µl supernatant) in the presence of increasing concentrations (10 µg, 1 µg, 100 ng and 10 ng) of wild-type or variant IL-6, as indicated on top of the lanes. After incubation and washing, the bound material was subjected to PAGE. M, molecular weight marker.
(**B**) Dimerization of sgp130 in the presence of wild-type and variants of IL-6. sgp130-myc was immobilized on protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with ^{35}S-labelled sgp130-FLAG (50 µl supernatant) in the presence of cold sIL-6Rα (500 µl supernatant) and increasing concentrations (10 µg, 1 µg and 100 ng) of wild-type or variants of IL-6, as indicated. After incubation and washing, the bound material was subjected to PAGE. M, molecular weight marker.

When the dimerization assay was performed using the protocol described in the previous section, all the IL-6 variants showed a selective and dramatic decrease (Figure 4B), in precise parallel with their impairment of bioactivity (Table I). In fact, DFRD, which does not possess residual bioactivity and is a full antagonist (Savino *et al.*, 1994b), does not dimerize gp130 at all, whereas variants W157R/D160R and T162D show a residual activity of ~3 and 1% respectively in HepG2 cells and are reduced in gp130 dimerization to ~6 and 4% respectively. From these results we can conclude that both site 2 and site 3 variants, while still maintaining the capability to recruit gp130 in the presence of IL-6Rα, cannot form gp130 dimers. This finding explains the lack of biological activity. What has to be taken into account, however, is that the DFRD mutation involving four amino acid residues is likely to completely abolish the interaction with the gp130 subunit binding at site 2, while the leaky mutations W157R/D160R and T162D do not fully impair the interaction of the respective gp130 subunits binding at site 3.

Apparently, the two areas of the IL-6 surface affected by these mutations behave as independent gp130 binding sites, because both site 2 and site 3 variants are able to bind one gp130 molecule efficiently. To analyse this further, two more variants bearing simultaneous mutations at sites 2 and 3 were generated (DFRD/W157R/D160R and DFRD/T162D). These variants maintain normal IL-6Rα binding (Table I) and, as expected, are biologically inactive. When tested for sgp130 binding, although with only slight differences, both variants were almost com-

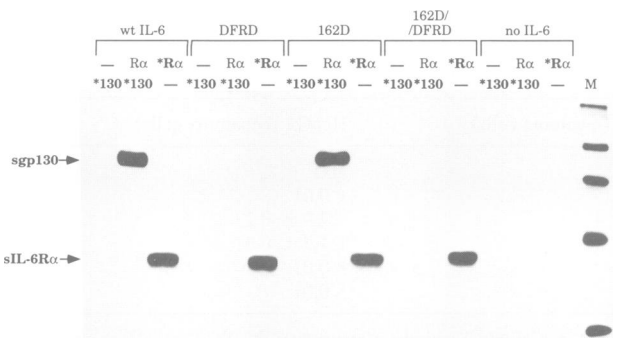

Fig. 5. Immunoprecipitation with mAb 16 of complexes formed with wild-type or variant IL-6. Wild-type or variant IL-6 (as indicated on top of the lanes) was immobilized on protein A–Sepharose beads using mAb 16 and incubated with the receptor molecules in the following amounts: 500 µl of supernatant containing cold sIL-6Rα and 50 µl of supernatant containing ^{35}S-labelled receptors. Rα, sIL-6Rα; 130, sgp130-FLAG; M, molecular weight marker. Asterisks indicate ^{35}S-labelled material. After incubation and washing, the bound material was subjected to PAGE.

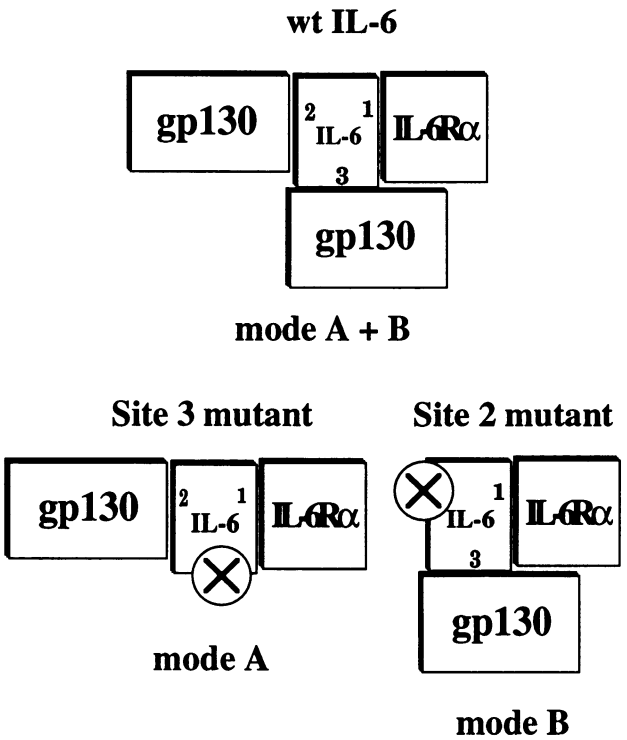

Fig. 6. Schematic representation of A and B modes of interaction of the IL-6Rα–IL-6 complex with gp130 molecules.

pletely unable to bind sgp130 (Figure 4A). The residual binding can be explained if we assume that residual binding still occurs at site 3 as discussed above. As expected, these variants did not dimerize gp130 (Figure 4B).

The 'two independent sites' hypothesis was tested further using the specific anti-IL-6 monoclonal antibody mAb 16 (Brakenhoff *et al.*, 1990). mAb 16 has been shown previously to inhibit the biological activity of IL-6 without interfering with its cell surface binding (Brakenhoff *et al.*, 1992). This led to the hypothesis that mAb 16 impairs the interaction of IL-6 with gp130 but not with IL-6Rα. The epitope recognized by mAb 16 was localized within the region between Q154 and T162, and this information was used for the development of site 3 IL-6 receptor antagonists (Brakenhoff *et al.*, 1992, 1994). In the light of these findings, we speculated that if IL-6 presents two independent binding sites for gp130, site 2 and site 3, the binding of mAb 16 could block only site 3, leaving site 2 free to interact with another gp130 molecule.

Therefore we performed immunoprecipitations with mAb 16 using wild-type or mutant IL-6, and tested their ability to bind and immunoprecipitate labelled IL-6Rα and sgp130. Initial results showed that of the site 3 mutations, W157R/D160R destroys the mAb 16 epitope whereas T162D can still be recognized by the antibody (data not shown).

As shown in Figure 5, all the variants tested bind sIL-6Rα efficiently, in agreement with the observation that mAb 16 does not interfere with this interaction. Moreover, mAb 16 still allowed wild-type IL-6 to interact efficiently with sgp130, promoting its co-immunoprecipitation. Not surprisingly, this interaction took place only in the presence of IL-6Rα, thus excluding the possibility that the neutralizing effect of this monoclonal antibody is due to a complete inhibition of gp130 interaction with the IL-6–IL-6Rα complex. Hence, the biological effect of mAb 16 is due to a selective inhibition of gp130 dimerization. Indeed, while the site 3-type T162D variant is still able to bind sgp130 in the presence of mAb 16, the site 2-type DFRD variant shows no residual binding to sgp130. Most probably, both sites are unavailable for this interaction, site 2 because of the mutation and site 3 because of the steric hindrance due to the bound antibody molecule.

In conclusion, by using a variety of tools it was possible to distinguish two independent modes of interaction of gp130 with the IL-6–IL-6Rα complex (outlined in Figure 6). In mode A one gp130 chain is oriented in a similar fashion to the second GHbp chain in the GH receptor complex (de Vos *et al.*, 1992); in mode B the second gp130 chain binds site 3 on IL-6 and is oriented differently to one side of the IL-6–IL-6Rα complex (Figure 6). In the presence of wild-type IL-6 both modes operate simultaneously and lead to gp130 homodimerization.

A variant in the E2 strand of the cytokine binding domain of IL-6Rα is selectively impaired in gp130 dimerization

Previous mutagenesis experiments with human IL-6Rα provided some information about the composition of the IL-6Rα–gp130 interface (Yawata *et al.*, 1993). Out of a large set of receptor variants, only a limited subset of amino acid substitutions in the IL-6Rα CBD was able to selectively reduce binding to gp130 while leaving the affinity for IL-6 intact. The majority of these mutations map in the E2 strand and AB2 loop of subdomain 2 of the receptor CBD, which is also the location of the binding interface between the two GH receptor chains in the GH–(GHbp)2 complex (de Vos *et al.*, 1992; Yawata *et al.*, 1993). Based on these findings we produced an IL-6Rα variant carrying two substitutions in the E2 strand (H280S/D281V) which was called Mut1. The construction of Mut1 and the characterization of its biochemical properties will be described elsewhere (A.L.Salvati, A.Lahm, G.Paonessa,

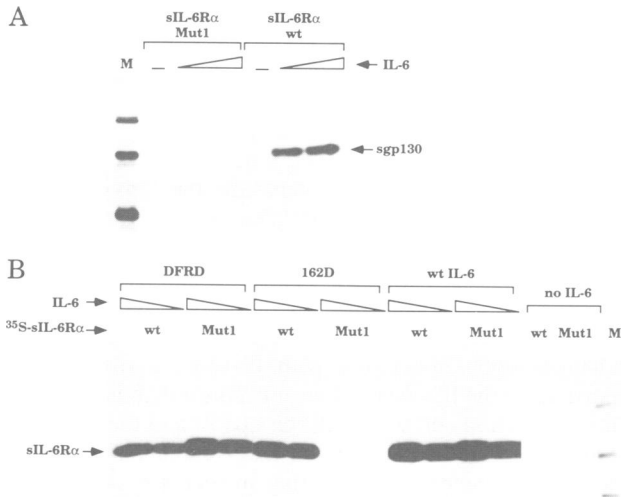

Fig. 7. The sIL-6Rα variant Mut1 has lost the ability to dimerize gp130. (**A**) Dimerization of sgp130 in the presence of sIL-6Rα variant Mut1. sgp130-myc was immobilized on protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with 50 μl of supernatant containing ^{35}S-labelled sgp130-FLAG and wild-type or variant sIL-6Rα (Mut1; 500 μl supernatant) in the absence and presence of 1 and 10 μg of wild-type IL-6, as indicated. After incubation and washing, the bound material was subjected to PAGE. M, molecular weight marker. (**B**) Binding of wild-type or variant sIL-6Rα (Mut1) in the presence of wild-type and variants of IL-6. sgp130-myc was immobilized on protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with sgp130-FLAG (500 μl supernatant) and 50 μl of supernatant containing ^{35}S-labelled wild-type or variant sIL-6Rα (Mut1) in the presence of 1 and 10 μg of wild-type and variants of IL-6, as indicated. After incubation and washing, the bound material was subjected to PAGE. M, molecular weight marker.

G.Ciliberto and C.Toniatti, manuscript in preparation). Mut1 is able to bind IL-6 with an affinity similar to that of wild-type IL-6Rα; therefore, the Mut1–wtIL-6 complex was tested for binding and its ability to promote dimerization of gp130. The results are shown in Figure 7A. Interestingly, Mut1 in combination with wild-type IL-6 still interacts with gp130 but displays a selective impairment in gp130 dimerization: this behaviour mirrors that of site 2 and site 3 IL-6 variants.

According to the GH–(GHbp)2-based trimeric model of the IL-6–IL-6Rα–gp130 interaction, the site defined by Mut1 in IL-6Rα is projected in the same orientation (and towards the same gp130 molecule) as site 2 on IL-6. This implies that gp130 cannot bind in mode A (Figure 6) to the Mut1–wtIL-6 complex, and that the residual binding is due to gp130 interactions in the B mode. As discussed before, this second gp130 appeared to be recruited in the complex with the contribution of site 3 in IL-6. To test this hypothesis we performed immunoprecipitation experiments combining Mut1 with a representative site 2 or site 3 variant. The results are shown in Figure 7B. In agreement with our predictions, the incubation of Mut1 with DFRD, which is a site 2 IL-6 variant, still results in binding to gp130. In contrast, although the site 3 T162D variant has normal binding affinity for Mut1 (data not shown), the complex formed by Mut1 and T162D has lost any residual binding to gp130: this result is similar to that obtained when the complex used is formed by wild-type IL-6Rα and a combined IL-6 site 2/3 variant (Figure 4B).

The IL-6 receptor complex is a hexamer which contains two IL-6, two IL-6Rα and two gp130 molecules

Structural comparisons and functional studies had suggested previously that the assembly of the IL-6 receptor complex requires the formation of an IL-6–IL-6Rα–gp130 heterotrimer which is structurally and topologically similar to the one assembled by GH–(GHbp)2 (Savino *et al.*, 1994a,b; Lahm *et al.*, 1995). The experiments presented here might indicate, however, that the functional complex is instead a tetramer, differing from the trimer by an additional binding of a second gp130 subunit with a 'novel' orientation. Alternatively, and most probably, it is possible that the functional complex has a different structure in which two trimeric complexes of the GH-type cluster close to each other to form a higher order symmetrical structure. To gain further insight into the stoichiometry of the IL-6 receptor complex, we designed immunoprecipitation experiments to test if *in vitro* complexes contain more than one IL-6Rα and/or IL-6 molecule.

To this end, Sepharose beads were coated with unlabelled myc-tagged sIL-6Rα (via anti-myc monoclonal antibody) and immunoprecipitations were performed in the presence of various combinations of IL-6, sgp130 (labelled or unlabelled) and [^{35}S]IL-6Rα. The results (Figure 8A) demonstrate that for IL-6Rα a second molecule is also recruited in the receptor complex in the presence of the other two components, namely IL-6 and sgp130.

Finally, to assess whether a second IL-6 molecule is also associated with the complex we used an IL-6 variant which bears the myc tag at its N-terminus (called hereafter IL-6myc). This molecule, produced in *Escherichia coli* and purified to homogeneity, is as biologically active as wild-type IL-6 (data not shown) and has the advantage of being specifically recognized by the anti-myc monoclonal antibody. The usual immunoprecipitation strategy was followed using cold IL-6myc as the 'first' IL-6 molecule, *in vitro* ^{35}S-labelled wild-type IL-6 as the 'second' IL-6 molecule and various combinations of receptors. As shown in Figure 8B, IL-6 also undergoes *in vitro* dimerization but only in the presence of both IL-6Rα and gp130. In conclusion, these experiments show that the incubation of IL-6 with the extracellular domains of IL-6Rα and gp130 causes the formation of complexes in which not only two gp130 subunits, but also two IL-6 molecules and two IL-6Rα subunits, are present.

Discussion

IL-6 stimulation of target cells causes covalent dimerization of the signal-transducing chain gp130, and these dimers have been found to be constantly associated with functional receptor complexes (Davis *et al.*, 1993; Murakami *et al.*, 1993). Although gp130 dimerization has been observed on cell membrane and therefore with the whole receptor molecule, we show here that this event is driven by the extracellular domains of both the α

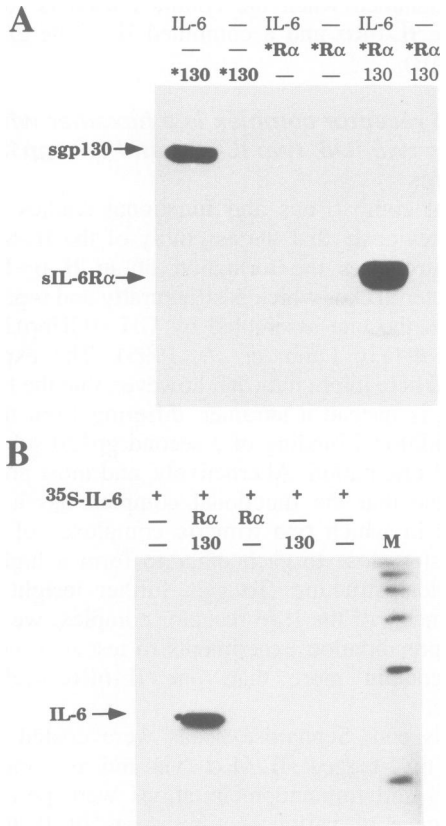

Fig. 8. Dimerization of IL-6Rα and IL-6. (**A**) Dimerization of IL-6Rα. sIL-6Rαmyc was immobilized on protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with the molecules indicated on the top of each lane in the following amounts: 3 μg IL-6, 500 μl supernatant containing cold sgp130-FLAG and 50 μl supernatant containing ^{35}S-labelled receptors. Rα, sIL-6Rα; 130, sgp130-FLAG. Asterisks indicate ^{35}S-labelled material. After incubation and washing, the bound material was subjected to PAGE. (**B**) Dimerization of IL-6. IL-6myc was immobilized on protein A–Sepharose beads via anti-myc 9E10 monoclonal antibody and incubated with the molecules indicated on the top of each lane in the following amounts: 50 μl ^{35}S-labelled IL-6, 500 μl supernatant containing cold sgp130-FLAG or sIL-6Rα. Rα, sIL-6Rα; 130, sgp130-FLAG; M, molecular weight marker. After incubation and washing, the bound material was subjected to PAGE.

(IL-6Rα) and β (gp130) receptor subunits because we observe efficient dimerization in the absence of both the transmembrane and cytoplasmic regions. We believe that these dimers faithfully reproduce what occurs on the cell surface, because their formation depends completely on the presence of IL-6–IL-6Rα complexes and they are not formed when IL-6 variants with normal binding affinity for IL-6Rα, but low or no biological activity, are used in place of wild-type IL-6.

The design of IL-6 receptor antagonists to be used for the treatment of IL-6 disregulation was approached with the idea that such molecules should possess normal (or improved) IL-6Rα binding affinity but impaired interaction with gp130 (Brakenhoff *et al.*, 1994; Savino *et al.*, 1994a,b). Interestingly, our results revealed that IL-6 receptor antagonists like DFRD (Savino *et al.*, 1994b) function through an unusual mechanism: they are able to recruit a single gp130 chain but fail to induce its homodimerization, thus lacking signalling capacity. Furthermore, their use in solution binding/immunoprecipitation reactions distinguished two distinct areas on IL-6 (sites 2 and 3), each of which is responsible for the independent binding of two gp130 chains with different orientations. This provides a molecular explanation for how the cytokine recruits two gp130 molecules, causes their dimerization and promotes signalling.

An important issue is what are the driving forces that bring about this process. Our study provides some important clues. Both site 2 and site 3 on IL-6 are unable to establish stable interactions with gp130 in the absence of IL-6Rα. Although we cannot exclude the possibility that these sites on IL-6 are 'activated' by conformational changes upon interaction with IL-6Rα, it may be that portions of the IL-6Rα surface are annexed, thus extending the interaction surfaces between gp130 and the IL-6–IL-6Rα complex. We favour the latter explanation because this is the mechanism operating in receptor assembly of GH, where it has been shown that when this hormone binds to its receptor, no major structural rearrangements occur (Ultsch *et al.*, 1994). Experiments using combinations of IL-6 variants in site 2 or site 3 and the receptor variant Mut1 strengthen our choice: site 2 in IL-6 plus residues 280/281 in IL-6Rα are projected towards the same gp130 molecule and together form a single combined site, whereas the other site is composed of a combination of site 3 and a still unidentified part of IL-6Rα. In each combined site the alteration of either the IL-6 or the IL-6Rα component causes loss of binding to the facing gp130 chain.

Immunoprecipitation experiments demonstrated that the receptor complex assembled *in vitro* contains not only two gp130 chains but also two IL-6 and two IL-6Rα molecules. We therefore propose that the active receptor complex is made of a hexamer with an IL-6–IL-6Rα–gp130 stoichiometry of 2:2:2. Based on previous 3-D modelling predictions and on the data presented in this paper, we present a schematic topological model for this new structure that can be called the 'hexameric IL-6 receptor complex'. The model is shown in Figure 9 both from a side view and projected along two planes. Two distinct regions have indeed to be defined in the hexamer: the top part in which the cytokine is located, and the bottom part formed by the second subdomains of the CBDs of both IL-6Rα and gp130 in which direct contacts are established by the receptor chains.

Although it is possible to consider the hexamer as formed by the clustering of two GH-like trimers juxtaposed in inverted orientations, we think that this is highly reductive because we believe that the hexamer is 'held' together by several interaction surfaces, some of which have already been identified and others of which can only be hypothesized. Many additional contacts could in fact be envisaged at both levels, for example direct interaction between the two IL-6 molecules. It is interesting to note that affinity cross-linking experiments on IL-6-responsive cells with iodinated IL-6 revealed the presence of IL-6 dimers, either alone or associated with an IL-6Rα molecule (Rose-John *et al.*, 1991; D'Alessandro *et al.*, 1993; Stoyan *et al.*, 1993). However, none of our site 2 and site 3 IL-6 variants map in the putative cytokine–cytokine contact surface because they do not show selective impairment of IL-6 dimerization in immunoprecipitation reactions (G.Paonessa and R.Graziani, unpublished results). From

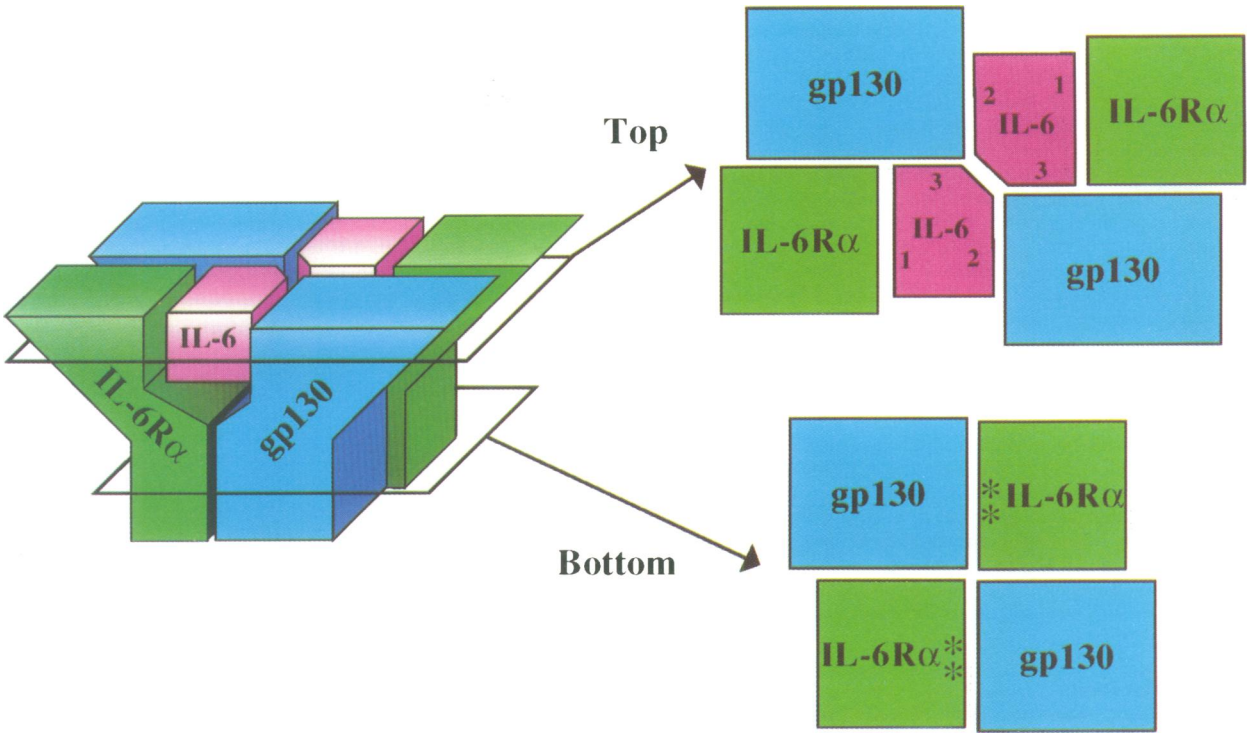

Fig. 9. Model of the hexameric IL-6 receptor complex. Shown are a side view of the IL-6 hexameric receptor complex (left) and two cross-sections through the complex (right): one that includes the bound cytokine (top), and a second (bottom) formed by the second subdomains of the CBDs of both IL-6Rα and gp130 where only interactions between the receptor molecules are postulated to occur. At this level, two asterisks in the IL-6Rα indicate the hypothetical location of the amino acids H280 and D281 mutagenized in the IL-6Rα variant Mut1. For IL-6, numbers identify the sites of interaction with the various receptor components (sites 1–3). The colour code is blue for gp130, green for IL-6Rα and pink for IL-6. For the receptors, only the part corresponding to their CBDs is shown.

the model, it could also be expected that gp130 has at least two sites of interaction with IL-6 and two with IL-6Rα, and targeted mutagenesis of the extracellular region of gp130 should be very useful in supporting this. Lastly, homodimerization contact surfaces between two gp130 and/or two IL-6Rα could be located at the bottom level formed by the second subdomains of the CBDs.

While this manuscript was being prepared it was reported that the incubation of purified IL-6−IL-6Rα complexes with soluble gp130 produced in CHO cells gave rise to complexes whose molecular weight is compatible only with the formation of hexamers with a 2:2:2 stoichiometry (Ward *et al.*, 1994). Therefore, identical conclusions have been obtained using a different approach. The use of wild-type molecules only did not, however, provide any information about the topological relationship of the various molecules in the assembled receptor complex (Ward *et al.*, 1994).

The hexamer is a novel type of receptor complex first discovered for IL-6. We propose, based on sequence homology data, that hexamers could be a common feature of receptor complexes assembled by other cytokines whose specific ligand binding receptors are predicted to be structurally and functionally similar to IL-6Rα. Among these are ciliary neurotrophic factor (CNTF) (Davis *et al.*, 1991) and probably also IL-11 (Hilton *et al.*, 1994). Indeed, immunoprecipitations performed using a strategy similar to that described in this paper for IL-6 strongly suggest that the dimerization of the CNTF−CNTF-R complex takes place in solution (A.De Serio, R.Graziani, R.Laufer, G.Ciliberto and G.Paonessa, manuscript in preparation). Interestingly, IL-12 (referred to also as natural killer cell stimulatory factor), which is by itself always found as a disulfide-linked heterodimer between a 35 kDa polypeptide related to IL-6 (Merberg *et al.*, 1992) and a 40 kDa polypeptide related to IL-6Rα (Gearing and Cosman, 1991), could also form a 2+2 receptor complex with its receptor subunit IL-12R which is strongly homologous to gp130 (Chua *et al.*, 1994). Because IL-6 binds first to a receptor chain that does not have any direct role in cell signalling, it is tempting to speculate that the active cytokine is indeed the IL-6−IL-6Rα complex; this is also supported by the evidence that soluble forms of IL-6Rα act as potentiators of cytokine activity (Taga *et al.*, 1989) and are present at high levels in the circulation (Honda *et al.*, 1992). Hexamer formation could thus be a common biological strategy which evolved to ensure the formation of high-affinity binding sites and firmly trap cytokines and/or growth factors into active configurations.

Materials and methods

Tagging and expression of the soluble receptors

Mutant cDNA coding for soluble forms of gp130 tagged at the C-terminus with myc (Evan *et al.*, 1985) or FLAG (Hopp *et al.*, 1988) epitopes were generated by inserting double-strand oligonucleotides at the *Eco*RI site of gp130 located around the codons for amino acids 605–606 (E−F). The resulting C-termini, were in the case of myc, E(605)-FEEQKLISEEDL-Stop (hereafter called sgp130-myc) and, in the case of FLAG, E(605)-FDYKDDDDK-Stop (hereafter called sgp130-FLAG). Mutant cDNA coding for soluble forms of IL-6Rα with and without myc tag epitope at the C-terminus were generated by PCR using suitable oligonucleotides. In the case of untagged sIL-6Rα (hereafter called sIL-6Rα), a stop codon was inserted after proline 322. In the case of

sIL-6Rα tagged with myc, myc epitope was inserted after proline 322 followed by a stop codon; the resulting C-terminus was P(322)-GGEQKLISEEDL-Stop (hereafter called sIL-6Rαmyc). The cDNAs of the soluble receptor variants sgp130-myc, sgp130-FLAG, sIL-6Rα and sIL-6Rαmyc were cloned into the *Bam*HI site of the baculovirus expression vector pBluBac III (Invitrogen). The generation of recombinant baculovirus expressing variants of gp130 and IL-6Rα was performed with MAXBAC kit (Invitrogen) according to the instructions of the manufacturer. The expression of cold receptors was accomplished by infecting High Five cells (Invitrogen) with a high titre stock of recombinant virus at a m.o.i. of 5–10. After 2 h the inoculum was removed and the cells were incubated at 27°C in SF-900 II medium (Gibco) until all cells detached from the plate (48–72 h). Medium was collected and centrifuged, and the supernatant was assayed by Western blot for recombinant protein production.

[^{35}S]Methionine labelling of recombinant receptors

Recombinant soluble receptors were metabolically labelled according to the following protocol. Semi-confluent High Five cells in a 6 cm plate were infected (m.o.i. of 5–10) with high titre recombinant baculovirus. After 36–48 h, medium was replaced with 2 ml of methionine-free Grace's insect medium and incubated at 27°C for 1 h. The medium was then replaced with 1.5 ml of methionine-free Grace's insect medium containing 50 μl of TRAN[^{35}S]LABEL (sp. act. >1000 Ci/mmol, 10 mCi/ml; ICN Biomedical Inc.) and incubated for a further 2 h. Medium was then collected, cell debris centrifuged and the supernatant used for the experiments.

Immunoprecipitations

Immunoprecipitation experiments were typically performed according to the following protocols. 500 μl baculovirus supernatant expressing myc-tagged receptors and 5 μg wild-type IL-6, variant IL-6 or IL-6myc were incubated with 4 μl anti-myc 9E10 monoclonal antibody (Evan *et al.*, 1985) or 1 μg monoclonal antibody IL-6-16 (CLB, The Netherlands) plus 40 μl 50% slurry in PBS of protein A–Sepharose (referred to hereafter as PAS; Pharmacia) overnight at 4°C. After washing three times with PBSTB (1× PBS, 0.05% Tween 20, 0.2% Brij 96), the Sepharose beads were incubated with other receptor(s) and/or cytokine (for further details see legends to the respective figures) for at least 12 h at 4°C. The beads were then washed three times with PBSTB, resuspended in SDS loading buffer, heated for 5 min at 95°C and subjected to SDS–PAGE.

Generation and expression of IL-6 variants

Generation and expression of IL-6 variants were performed exactly as described previously (Savino *et al.*, 1994b). For the amino acid numbering of IL-6 see Savino *et al.* (1994a). IL-6 tagged with myc epitope at the N-terminus (IL-6myc) was generated by PCR using suitable oligonucleotides; it was cloned in pT7.7 vector (Studier *et al.*, 1990). The resulting sequence of the N-terminus is the following: Met-EQKLISEEDL-wtIL-6, where the first amino acid of IL-6 is represented by valine in position 2. ^{35}S-labelling of wild-type IL-6 was performed with rabbit reticulocyte lysate (Promega) according to the supplier's instructions.

In vitro receptor binding assays and bioassays

In vitro receptor binding assays and bioassays on HepG2 and XG-1 cell lines were performed exactly as described previously (Savino *et al.*, 1994b).

Acknowledgements

We thank Anna Tramontano, Maurizio Sollazzo, Ralph Laufer and Janet Clench for critically reading this manuscript and Yves Cully and Manuela Emili for artwork.

References

Bazan,J.F. (1990a) Hemapoietic receptors and helical cytokines. *Immunol. Today*, **11**, 350–354.

Bazan,J.F. (1990b) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl Acad. Sci. USA*, **87**, 6934–6938.

Bazan,J.F. (1991) Neuropoietic cytokines in the hematopoietic fold. *Neuron*, **7**, 197–208.

Brakenhoff,J.P.J., Hart,M., de Groot,E.R., Di Padova,F. and Aarden,L.A. (1990) Structure–function analysis of human IL-6. *J. Immunol.*, **145**, 561–568.

Brakenhoff,J.P.J., de Hon,F.D., Fontaine,V., Hart,M., de Groot,E.R., Content,J. and Aarden,L.A. (1992) Two different sites on the IL-6 molecule are involved in biological activity. In Revel,M. (ed.), *IL-6: Physiopathology and Clinical Potential.* Serono Symposium Publication, Raven Press, New York, Vol. 88, pp. 33–41.

Brakenhoff,J.P.J., de Hon,F.D., Fontaine,V., Boekel,E.T., Schooltink,H., Rose-John,S., Heinrich,P.C., Content,J. and Aarden,L.A. (1994) Development of a human interleukin-6 receptor antagonist. *J. Biol. Chem.*, **269**, 86–93.

Carson, M. (1987) Ribbon models of macromolecules. *J. Mol. Graphics*, **5**, 103–106.

Chua,A.O. *et al.* (1994) Expression cloning of a human IL-12 receptor component. A new member of the cytokine receptor superfamily with strong homology to gp130. *J. Immunol.*, **153**, 128–136.

Cosman,D. (1993) The hematopoietin receptor superfamily. *Cytokine*, **5**, 95–106.

D'Alessandro,F., Colamonici,O.R. and Nordan,R.P. (1993) Direct association of interleukin-6 with a 130-kDa component of the interleukin-6 receptor system. *J. Biol. Chem.*, **268**, 2149–2153.

Davis,S., Aldrich,T.H., Valenzuela,D.M., Wong,V., Furth,M.E., Squinto, S.P. and Yancopoulos,G.D. (1991) The receptor for ciliary neurotrophic factor. *Science*, **253**, 59–63.

Davis,S., Aldrich,T.H., Stahl,N., Pan,L., Taga,T., Kishimoto,T., Ip,N.Y. and Yancopoulos,G.D. (1993) LIFRβ and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science*, **260**, 1805–1808.

de Vos,A.M., Ultsch,M. and Kossiakoff,A.A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science*, **255**, 306–312.

Ehlers,M., Grötzinger,J., deHon,F.D., Müllberg,J., Brakenhoff,J.P.J., Liu,J., Wollmer,A. and Rose-John,S. (1994) Identification of two novel regions of human IL-6 responsible for receptor binding and signal transduction. *J. Immunol.*, **153**, 1744–1753.

Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. *Mol. Cell. Biol.*, **5**, 3610–3616.

Gearing,D.P. and Cosman,D. (1991) Homology of the p40 subunit of Natural Killer Cell Stimulatory Factor (NKSF) with the extracellular domain of the interleukin-6 receptor. *Cell*, **66**, 9–10.

Hibi,M., Murakami,M., Saito,M., Hirano,T., Taga,T. and Kishimoto,T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell*, **63**, 1149–1157.

Hilton,D.J. *et al.* (1994) Cloning of a murine IL-11 receptor α-chain; requirement for gp130 for high affinity binding and signal transduction. *EMBO J.*, **13**, 4765–4775.

Honda,M., Yamamoto,S., Cheng,M., Yasukawa,K., Suzuki,H., Saito,T., Osugi,Y., Tokunaga,T. and Kishimoto,T. (1992) Human soluble IL-6 receptor: its detection and enhanced release by HIV infection. *J. Immunol.*, **148**, 2175–2180.

Hopp,T.P., Prickett,K.S., Price,V.L., Libby,R.T., March,C.J., Cerretti,D.P., Urdal,D.L. and Conlon,P.J. (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio/Technology*, **6**, 1204–1210.

Lahm,A. *et al.* (1995) The molecular design of human IL-6 receptor antagonists. *Ann. NY Acad. Sci.*, **762**.

Merberg,D.M., Wolf,S.F. and Clark,S.C. (1992) Sequence similarity between NKSF and the IL-6/G-CSF family. *Immunol. Today*, **13**, 77–78.

Murakami,M., Hibi,M., Nakagawa,N., Nakagawa,T., Yasukawa,K., Yamanishi,K., Taga,T. and Kishimoto,T. (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science*, **260**, 1808–1810.

Rose-John,S., Hipp,E., Lenz,D., Legrés,L.G., Korr,H., Hirano,T., Kishimoto,T. and Heinrich,P.C. (1991) Structural and functional studies on the human interleukin-6 receptor. *J. Biol. Chem.*, **266**, 3841–3846.

Savino,R., Lahm,A., Giorgio,M., Cabibbo,A., Tramontano,A. and Ciliberto,G. (1993) Saturation mutagenesis of the human interleukin 6 receptor-binding site: Implications for its three-dimensional structure. *Proc. Natl Acad. Sci. USA*, **90**, 4067–4071.

Savino,R., Lahm,A., Salvati,A.L., Ciapponi,L., Sporeno,E., Altamura,S., Paonessa,G., Toniatti,C. and Ciliberto,G. (1994a) Generation of interleukin-6 receptor antagonists by molecular-modelling guided mutagenesis of residues important for gp130 activation. *EMBO J.*, **13**, 1357–1367.

Savino,R., Ciapponi,L., Lahm,A., Demartis,A., Cabibbo,A., Toniatti,C.,

Delmastro,P., Altamura,S. and Ciliberto,G. (1994b) Rational design of a receptor super-antagonist of human interleukin-6. *EMBO J.*, **13**, 5863–5870.

Sporeno,E., Paonessa,G., Salvati,A.L., Graziani,R., Delmastro,P., Ciliberto,G. and Toniatti,C. (1994) Oncostatin M binds directly to gp130 and behaves as interleukin-6 antagonist on a cell line expressing gp130 but lacking functional oncostatin M receptors. *J. Biol. Chem.*, **269**, 10991–10995.

Stahl,N. *et al.* (1994) Association and activation of Jak-Tyk kinases by CNTF–LIF–OSM–IL-6 beta receptor components. *Science*, **263**, 92–95.

Stoyan,T., Michaelis,U., Schooltink,H., Van Dam,M., Rudolph,R., Heinrich,P.C. and Rose-John,S. (1993) Recombinant soluble interleukin-6 receptor: expression in *Escherichia coli*, renaturation and purification. *Eur. J. Biochem.*, **216**, 239–245.

Studier,F.W., Rosemberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.*, **185**, 60–89.

Taga,T., Hibi,M., Hirata,Y., Yamasaki,K., Yasukawa,K., Matsuda,T., Hirano,T. and Kishimoto,T. (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell*, **58**, 573–581.

Ultsch,M.H., Somers,W., Kossiakoff,A.A. and de Vos,A.M. (1994) The crystal structure of affinity-maturated human Growth Hormone at 2 Å resolution. *J. Mol. Biol.*, **236**, 286–299.

Van Snick,J. (1990) Interleukin-6: an overview. *Annu. Rev. Immunol.*, **8**, 253–278.

Ward,L.D., Howlett,G.J., Discolo,G., Yasukawa,K., Hammacher,A., Moritz,R. and Simpson,R.J. (1994) High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. *J. Biol. Chem.*, **269**, 23286–23289.

Yamasaki,K., Taga,T., Hirata,Y., Yawata,H., Kawanishi,Y., Seed,B., Taniguchi,T., Hirano,T. and Kishimoto,T. (1988) Cloning and expression of the human interleukin-6 (BSF-2/IFNβ2) receptor. *Science*, **241**, 825–828.

Yawata,H., Yasukawa,K., Shunji,N., Murakami,M., Yamasaki,K., Hibi,M., Taga,T. and Kishimoto,T. (1993) 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.

Zhong,Z., Wen,Z. and Darnell,J.E.,Jr (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*, **264**, 95–98.

Received on January 20, 1995