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Soluble IL-6 Receptor Potentiates the Antagonistic Activity of Soluble gp130 on IL-6 Responses

Gerhard Müller-Newen,* Andrea Küster,* Ulrike Hemmann,* Radovan Keul,* Ursula Horsten,* Astrid Martens,* Lutz Graeve,* John Wijdenes,† and Peter C. Heinrich2*

Soluble receptors for several cytokines have been detected in body fluids and are believed to modulate the cytokine response by binding the ligand and thereby reducing its bioavailability. In the case of IL-6, the situation is more complex. The receptor consists of two components, including a ligand-binding α-subunit (IL-6R, gp80, or CD126), which in its soluble (s) form (sIL-6R) acts agonistically by making the ligand accessible to the second subunit, the signal transducer gp130 (CD120b). Soluble forms of both receptor subunits are present in human blood. Gel filtration of iodinated IL-6 that had been incubated with human serum revealed that IL-6 is partially trapped in IL-6/sIL-6R/gp130 ternary complexes. sgp130 from human plasma was enriched by immuno-affinity chromatography and identified as a 100-kDa protein. Functionally equivalent rsgp130 was produced in baculovirus-infected insect cells to study its antagonistic potential on different cell types. It was found that in situations in which cells lacking membrane-bound IL-6R were stimulated with IL-6/sIL-6R complexes, sgp130 was a much more potent antagonist than it was on IL-6R-positive cells stimulated with IL-6 alone. In the latter case, the neutralizing activity of sgp130 could be markedly enhanced by addition of sIL-6R. As a consequence of these findings, sIL-6R of human plasma must be regarded as an antagonistic molecule that enhances the inhibitory activity of sgp130. Furthermore, in combination with sIL-6R, sgp130 is a promising candidate for the development of IL-6 antagonists. The Journal of Immunology, 1998, 161: 6347–6355.

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Materials and Methods

\textbf{Enzymes, proteins, Abs, chemicals, and cell culture media}

Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), and protein A-Sepharose was purchased from Pharmacia (Freiburg, Germany). DMEM, DMEM/F12, RPMI 1640, SF-900 II medium, and antibiotics were obtained from Life Technologies (Eggenstein, Germany), and streptomycin was obtained from Seromed (Berlin, Germany). Biotinyl-Hunter reagent and [α-32P]dATP were purchased from Amersham International (Little Chalfont, U.K.), and Tran[α-32P]S] label metabolic labeling reagent was purchased from ICN (Meckenheim, Germany). Recombinant human IL-6 was expressed in \textit{Escherichia coli}, refolded, and purified as described by Arcone et al. (22). The specific activity was \(10^8\) units/mg of protein in the solution (10-100 ng/well) and incubated for 45 min at room temperature. After a final wash, substrate rsgp130. Subsequently, the plates were washed again, and streptavidin 37°C. The standard curve was obtained by twofold serial dilutions of ylated mAb (B-T2, 50 ng/well) were incubated simultaneously for 2 h. The reaction mixtures were subjected to gel filtration column, and 0.003% H2O2 was added. After incubation for 30 min in the dark, the color reaction was stopped with 2 M H2SO4, and the absorbance at 450 nm was determined using an ELISA reader (T-Lab Instruments, Grödig, Austria). Affinity-purified rsgp130 from baculovirus-infected insect cells was used as a standard.

\textbf{Construction of the recombinant baculoviruses and expression of rsgp130}

The Acc\textit{H}-EcoRI fragment encoding the gp130 extracellular domains (codons 1-606) was cut out from the vector pVL-gp130. A pair of hydridized oligonucleotides (5'-AA TTC GGA (CAT), CAC TAG-3' and 5'-G ATC CTA GTG (ATG) 5 TCC G-3) encoding a glycine and six histidines was used to introduce a HindIII site into the expression vector. A HindIII site was linked to the EcoRI site of the cDNA for soluble human gp130 and inserted into the polyhedrin locus-based baculovirus transfer vector pVL1392 using the BglII (blunt end) and the BamHI sites. SF158 cells were cotransfected with 0.5 \mu g of recombinant gp130 baculovirus transfer vector and 0.125 \mu g of Baculovirus DNA as outlined in the BaculoGold transfection kit (BaculoGold) and grown in Sf9 cells. A single virus clone was determined by end point dilution. Several clones were screened for expression of gp130 by Western blotting. The selected virus clone was then amplified by infecting SF158 cells at a multiplicity of infection less than 1. SF158 insect cells were grown at 27°C as monolayer cultures in serum-free SF-900 II medium. For protein expression, exponentially growing cells were infected with the rsgp130 baculovirus in suspension cultures at a multiplicity of infection from 10 to 20. Seventy-two hours after infection, the cells and cellular debris were sedimented by centrifugation, and the culture supernatants were stored at -20°C.

\textbf{Immunoadfinity purification of sgp130 and rsgp130}

The gp130 mAb B-T12 was coupled to 0.5 g of CNBr-activated Sepharose CL-4B according to the protocol of the supplier (Pharmacia). Three milliliters of 1 M CaCl2 was added to 300 ml of human plasma, and after incubation at 37°C for 90 min, 10 ml of 0.5 M EDTA was added. Coagulated proteins were sedimented by centrifugation for 20 min at 10,000 \times g. The supernatant was applied to the immunoadfinity column at a flow rate of 20 ml/h. To purify rsgp130, supernatants of baculovirus-infected insect cells were loaded onto the column. After washing with 100 ml PBS/0.05% Tween 20, 200 ml PBS/rsgp130 was eluted with 0.2 M glycine buffer, pH 2.5, and immediately neutralized with 1 M Tris/HC1, pH 8.0. Pooled fractions were dialyzed against PBS, and the gp130 concentration was determined by ELISA. Total protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

\textbf{Gel filtration}

Analyses of complexes formed by iodinated IL-6 in human serum were performed on a calibrated Superdex 200 16/60 gel filtration column (Pharmacia) at a flow rate of 0.85 ml/min. SL-6R depletion of human serum was performed as described (26). 125I-labeled IL-6 (20 ng) with a specific activity of 4.7 \times 10^7 cpm/ng was incubated with 1 ml of human serum (either normal or SL-6R depleted) or 1 ml of a solution of BSA (50 mg/ml) in PBS for 2 h at 37°C. After centrifugation for 10 min at 10,000 \times g, the supernatant was applied to the gel filtration column, and 0.2-ml fractions were collected. Immunoprecipitations were performed using B-P4-saturated protein A-Sepharose. 125I-labeled IL-6 was quantified using a gamma counter.

\textbf{Formation of ternary complexes of IL-6, sIL-6R, and sgp130}

Binding experiments were performed using IL-6 radiolabeled with 125I according to the procedure of Bolton and Hunter (27), affinity-purified sIL-6R, and rsgp130 or conditioned media from SF158 cells expressing rsgp130. All incubations were conducted at 4°C. 125I-labeled IL-6 (5.5 ng) with a specific activity of 8.3 \times 10^7 cpm/ng was preincubated overnight with 100 ng sIL-6R in 500 \mu l TNET buffer (20 ml Tris-HCl, pH 7.5; 140 mM NaCl; 5 mM Na2EDTA; 1% Triton X-100; 2 mM methionine; and 0.01% NaN3). Increasing amounts of purified rsgp130 or conditioned medium containing rsgp130 were added, and the incubation was continued for 2 h. The reaction mixtures were subjected to immunoprecipitation with the monoclonal gp130 Ab B-T12. Coprecipitated 125I-labeled IL-6 was quantified using a gamma counter.

\textbf{Real-time interaction analysis}

For direct monitoring of ternary complex formation, the IAgsys (Fisons, Cambridge, U.K.) system was used. The biosensor surface was activated using N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carboimide and subsequently incubated with 100 ng Ab B-T12 (50 \mu g/ml in 10 mM sodium acetate, pH 4.8) for 8 min. Unbound Abs were removed by several washes with PBS containing 0.05% Tween 20 (PBS-T), and residual activated groups were blocked with 1 M ethanolamine, pH 8.5. Supernatants of baculovirus-infected insect cells expressing rsgp130 (40 \mu l) were diluted 5-fold with PBS-T and added to the cuvette. Subsequently, the cuvette was incubated with IL-6 (10 \mu g/ml in PBS-T), sIL-6R (10 \mu g/ml in PBS-T), and IL-6 plus sIL-6R (5 \mu g/ml each in PBS-T) (time intervals indicated in Fig. 4A). After each exchange of protein solution, the cuvette was rinsed with PBS-T. Binding events were monitored as an increase of the resonance angle α.

\textbf{Ba/F3 proliferation assay}

Ba/F3-gp130 (28) cells were cultured in RPMI 1640 containing 10% FCS, plated on 96-well plates (20,000 cells/well), and stimulated with either IL-6/sIL-6R or 5% (v/v) conditioned medium from X63Ag-653 BPV-mIL-3 myeloma cells (as a source of IL-3) in the presence of rsgp130 (concentrations indicated in Fig. 5). After 60 h of incubation, viable and metabolically active cells were quantified using a colorimetric assay based on the Cell Proliferation Kit II sodium 3-(4,5-dimethylthiazol-2-yl)-5-(phenylaminocarbonyl)-3.2-tetraolium-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay (Boehringer Mannheim).

\textbf{Analysis of STAT activation in COS-7 and Madin-Darby canine kidney (MDCK)/IL-6R cells by electrophoretic mobility shift assay (EMSA)}

COS-7 and MDCK cells were cultured in DMEM containing 10% FCS. The cDNA of IL-6R was subcloned into the expression vector pCB8 (kindly provided by A. Le Briv, Laboratoire de Genetique et Physiologie de Developpement, Faculte des Sciences, Marseille, France). Transfection of MDCK cells was performed by a modification of the calcium phosphate precipitation procedure described by Graham and van der Eb (29). Resistant cells growing in the presence of 0.5 mg/ml G418 (Life Technologies) for 14 days were screened for IL-6R expression by indirect immunofluorescence and binding of radiiodinated IL-6. To induce IL-6R expression, the MDCK/KC/IL-6R cells were treated with 10 mM sodium butyrate (Sigma, St. Louis, MO) for 15 h, and COS-7, MDCK, or MDCK/KC/IL-6R cells were incubated at 37°C for 15 min in the presence of IL-6, rsgp130, and sIL-6R (concentrations as indicated in Figs. 6 and 9). Preparation of nuclear extracts and EMSAs were performed as described (30). A mutated double-stranded oligonucleotide corresponding to the c-fos promoter (m67SIE, 5'-GAT CCG GGA GGG ATT TAC GGG GAA ATG CGT-3'), which provides STAT3 and STAT1 binding sites, was used as a 32P-labeled probe. Hotel nuclear complexes were separated on a 4.5% polyacrylamide gel.
containing 7.5% glycerol in 23 mM Tris/23 mM boric acid, pH 8.0, and 0.5 mM EDTA at 20 V/cm for 4 h. Gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid, and 80% (v/v) water for 30 min; dried; and analyzed by autoradiography.

**Induction of acute-phase protein synthesis in HepG2 cells**

HepG2 cells were incubated in DMEM/F12 with IL-6, rsgp130, and sIL-6R (concentrations as indicated in Fig. 7) for 18 h and metabolically pulse labeled with [35S]methionine for 3 h. Induction of the newly synthesized acute-phase protein α1-antichymotrypsin was measured in cell culture supernatants by immunoprecipitation using a rabbit anti-human α1-antichymotrypsin antiserum. Immunocomplexes were precipitated with protein A-Sepharose, separated on 10% SDS-polyacrylamide gels, and visualized by autoradiography.

**Western blotting**

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes by a semidry blotting procedure (31). The membranes were incubated with the Ab mixtures as indicated in the figures and were processed for chemiluminescence detection as described in the enhanced chemiluminescence manual (Amersham International).

**Results**

**Establishment of an ELISA for the quantification of sgp130**

To investigate the role of sgp130 in the modulation of IL-6 responses in a quantitative manner, an sgp130 ELISA was established. Several mAbs (described in Ref. 25) directed against the ectodomain of gp130 were tested for their usefulness in a sandwich ELISA. The best results regarding sensitivity and dose response were obtained by the use of B-P4 for coating of the microtiter plate and biotinylated B-T2 for the detection of sgp130. Concentrations between 100 pg/ml and 2000 pg/ml were quantified using purified rsgp130 as a standard (not shown). Plasma levels of sgp130 determined with our ELISA (320 ± 622 ng/ml, n = 6) are in good agreement with those previously reported (390 ± 70 ng/ml; Ref. 20). The ELISA can also be used for the detection of sgp130 complexed with IL-6/sIL-6R (see below).

**IL-6 added to human serum forms a high molecular mass complex with sgp130 that depends on the presence of sIL-6R**

To prove the possible functional role of sgp130 in the modulation of IL-6 responses, [125I]-labeled IL-6 was added to human serum, and the protein complexes formed were analyzed by gel filtration. A substantial portion of [125I]-labeled IL-6 appeared in a peak covering a molecular mass range of 450–100 kDa (Fig. 1A, closed circles, fractions 25–29). An additional smaller peak of [125I]-labeled IL-6 eluted with the void volume of the column. Both peaks were not detected when [125I]-labeled IL-6 was incubated with physiologic concentration of serum albumin in PBS and then subjected to gel filtration (Fig. 1A, closed triangles). The high background levels seen between fractions 22 and 31 may be due to association of [125I]-labeled IL-6 with different oligomeric forms of BSA. Analysis of the serum fractions by ELISA revealed that sgp130 comigrated with the 450–100-kDa [125I]-labeled IL-6 peak (Fig. 1B, open circles). Immunoprecipitation of proteins in fractions 25–28 containing sgp130 using the sgp130 mAb B-P4 led to the coprecipitation of [125I]-labeled IL-6 (Fig. 1C, fractions 25–28, solid bars) indicated by arrows; V₀, void volume. B, Elution profile of sgp130 in the gel filtration experiments described in A as determined by ELISA (○, normal human serum; □, sIL-6R-depleted human serum). C, The fractions of the gel filtration experiment described in (A) that contained sgp130 (25–28) and fraction 36 as a control were subjected to immunoprecipitation with the sgp130 mAb B-P4. Coprecipitated radioactivity due to association of [125I]-labeled IL-6 is depicted as a percentage of total radioactivity of the corresponding fraction (solid bars, normal human serum; gray bars, sIL-6R-depleted human serum).
SGP130 mAb was observed in the low molecular mass 125I-labeled fractions 25–27. No precipitation of 125I-labeled IL-6 with the fractions corresponding to a molecular mass of 450–200 kDa IL-6. The highest portion of radioactivity was precipitated in the indicative of a physical association of sgp130 and 125I-labeled IL-6. The highest portion of radioactivity was precipitated in the fractions corresponding to a molecular mass of 450–200 kDa (fractions 25–27). No precipitation of 125I-labeled IL-6 with the sgp130 mAb was observed in the low molecular mass 125I-labeled IL-6 peak (Fig. 1C, fraction 36, solid bar).

The same experiment was performed with serum, which was previously sIL-6R depleted by incubation with IL-6-Sepharose (Fig. 1A, closed squares). The amount of radioactivity in the 450–100-kDa peak was reduced from 12.5% of total radioactivity in the presence of sIL-6R to 5.3% in the absence of sIL-6R. Reduction of radioactivity was most pronounced in the fractions corresponding to a molecular mass of 450–200 kDa. The portion of radioactivity that was precipitated with the sgp130 Ab was in the range of only 1% of total radioactivity of the corresponding fraction (Fig. 1C, gray bars). However, in the presence of sIL-6R, up to 6.6% of radioactivity was precipitated (Fig. 1C, solid bars). In the absence of sIL-6R, the sgp130 peak was moderately shifted to lower molecular mass (Fig. 1B, open squares). We conclude from these experiments that the sgp130 peak obtained after gel filtration of plasma proteins in the presence of radiolabeled IL-6 in its high molecular mass part (fractions 25–27) contains IL-6/sIL-6R/sgp130 ternary complexes and in its low molecular mass part (fractions 27–30) consists predominantly of sgp130.

Characterization of sgp130 from human plasma

To characterize sgp130 from human plasma, the protein was enriched by immunoaffinity chromatography. Clotting of human plasma was induced by the addition of Ca2+ ions, and precipitated fibrin was separated by centrifugation. No major loss of sgp130 was observed by the coagulation process as determined by ELISA. The clear supernatant was loaded onto a column of Sepharose-bound gp130 mAb B-T12. The column was rinsed with PBS-Tween, and bound proteins were eluted with 0.2 M glycine buffer, pH 2.5. Fractions of 1 ml were collected and neutralized with Tris-HCl buffer, pH 8.0. sgp130 concentrations of the fractions were determined by ELISA.

FIGURE 2. Immunoaffinity purification of sgp130 from human plasma. A, Elution profile of sgp130 immunoaffinity chromatography. Human plasma (7 ml) was loaded onto a column of Sepharose-bound gp130 mAb B-T12. The column was rinsed with PBS-Tween, and bound proteins were eluted with 0.2 M glycine buffer, pH 2.5. Fractions of 1 ml were collected and neutralized with Tris-HCl buffer, pH 8.0. sgp130 concentrations of the fractions were determined by ELISA. B, Enriched sgp130 after immunoaffinity chromatography (50 ng) was subjected to SDS-PAGE. Proteins in lane M (marker proteins) and lane 1 were silver stained. Lane 2 shows an immunoblot analysis using the monoclonal sgp130 Ab B-P4.

Expression of human sgp130 in baculovirus-infected insect cells and characterization of the recombinant protein

Since the available amount of sgp130 from human plasma was too low, we produced human rsgp130 for further studies. For this purpose, insect cells were infected with a recombinant baculovirus encoding the human gp130 ectodomain (amino acids 1–606 followed by a polyhistidine tag; for details see Materials and Methods). After 3 days, the highest concentration of rsgp130 was observed: about 3 μg/ml as determined by ELISA. This corresponds to about 1% of total protein (250–300 μg/ml). Furthermore, the immunoblot revealed an apparent molecular mass of about 65 kDa.

FIGURE 3. Immunoaffinity purification of rsgp130 from supernatants of baculovirus-infected insect cells. Conditioned medium (20 μl; lane 1) and purified rsgp130 (100 ng; lane 2) were subjected to SDS-PAGE followed by silver staining. M, marker proteins.
for rsgp130 (not shown), suggesting a much lower extent of glycosylation compared with sgp130 from human plasma. Supernatants collected 3 days postinfection were used for affinity purification of rsgp130 by the same method as described for sgp130 from human plasma. Analysis of the fractions by SDS-PAGE revealed that rsgp130 eluted as a pure protein (Fig. 3, lane 2). Purification, however, was accompanied by a marked loss of recombinant protein. Freshly prepared rsgp130 eluted from the gel filtration column as a monomer (not shown).

The binding capability of rsgp130 from baculovirus-infected insect cells was demonstrated using two different approaches. In Fig. 4A, a surface plasmon resonance experiment is shown, in which binding events are measured in real time as an increase of the resonance angle \( \alpha \). The sgp130 mAb B-T12 was covalently linked to the sensor surface and subsequently incubated with insect cell supernatants containing rsgp130, resulting in an increase of the resonance angle due to binding of sgp130 to the Ab. Addition of IL-6 alone did not lead to any binding event, whereas addition of sIL-6R resulted in a weak increase of the resonance angle, possibly due to a very low affinity binding of sIL-6R to rsgp130. Addition of a combination of IL-6 and sIL-6R led to the expected strong binding event. Similar results were obtained by coimmunoprecipitation of \(^{125}\)I-labeled IL-6 (Fig. 4B). Using the sgp130 mAb B-T12, IL-6 was not precipitated in the presence of rsgp130 or in the presence of sIL-6R alone. Only the combination of rsgp130 and sIL-6R led to the coprecipitation of IL-6. The amount of coprecipitated IL-6 corresponded to the increasing amounts of sgp130 used. The binding assay worked with freshly purified rsgp130, as well as with conditioned insect cell medium containing rsgp130.
rsgp130 efficiently inhibits IL-6/sIL-6R-induced responses on cells lacking membrane-bound IL-6R

Ba/F3 cells, which constitute a pre-B cell line often used for the study of cytokine responses, grow in the presence of IL-3. After transfection with gp130, these cells become IL-6/sIL-6R responsive, but due to the lack of membrane-bound IL-6R, they do not respond to IL-6 alone. A previously established Ba/F3-gp130 cell line (28) was used to analyze the antagonistic potential of rsgp130. Ba/F3 cells were incubated with IL-3 or IL-6 and sIL-6R in the presence of increasing amounts of rsgp130. The response of the Ba/F3-gp130 cells to IL-3 was not affected by rsgp130 (Fig. 5, circles), whereas the response to IL-6/sIL-6R was suppressed in a dose-dependent manner (Fig. 5, squares). Proliferation of the cells incubated with rsgp130 alone (Fig. 5, triangles) were indistinguishable from unstimulated control cells (not shown). In the presence of 10 ng/ml IL-6 and 1 μg/ml sIL-6R, 1 μg/ml sgp130 was sufficient for the complete inhibition of the IL-6 signal.

To test the influence of rsgp130 on downstream signaling events, STAT activation in COS-7 cells was analyzed. Since COS-7 cells endogenously express gp130 but no membrane-bound IL-6R, again stimulation with IL-6/sIL-6R was required. The EMSA presented in Fig. 6 shows that incubation of COS-7 cells with IL-6/sIL-6R (10 ng/ml and 1 μg/ml, respectively) resulted in a strong STAT1 activation, which is typical for this cell type (Fig. 6, lane 1). Whereas the presence of control supernatant had no significant effect on the signal intensity (Fig. 6, lane 2), STAT activation was completely abolished by the addition of supernatant containing rsgp130 (final concentration in the assay, 1 μg/ml; Fig. 6, lane 3). Thus, on cells lacking membrane-bound IL-6R, the stimulation with IL-6/sIL-6R can efficiently be blocked by rsgp130.

In the presence of rsgp130, the sIL-6R acts antagonistically on cells expressing membrane-bound IL-6R by increasing the inhibitory effect of rsgp130

Next, we investigated the antagonistic activity of rsgp130 on cells expressing membrane-bound IL-6R. These cells respond to IL-6 without the requirement of sIL-6R. HepG2 cells, a human hepatoma cell line, respond to IL-6 with the synthesis of the acute-phase protein α1-antichymotrypsin, which, after metabolic labeling, can be immunoprecipitated from cell supernatants and detected by autoradiography (Fig. 7, lane 6). Here, we repeatedly observed that amounts of rsgp130 (1 μg/ml) that were sufficient to completely suppress the IL-6 signal on Ba/F3-gp130 or COS-7 cells showed only a weak antagonistic effect on HepG2 cells (Fig. 7, lane 9). Most surprisingly, sIL-6R, which normally acts agonistically on HepG2 cells (Fig. 7, lane 7), in combination with rsgp130 increased the antagonistic effect of rsgp130 (Fig. 7, lane 8). We repeated this experiment with physiologic concentrations of IL-6, sIL-6R, and sgp130. Again, an inhibitory effect of sIL-6R in the presence of sgp130 was observed (Fig. 7, lanes 1–5). Due to the lack of membrane-bound IL-6R, MDCK cells do not respond to IL-6 (Fig. 8, lane 5). After stable transfection with a cDNA encoding membrane-bound IL-6R, these cells become IL-6 responsive, resulting in a strong activation of STAT3 and STAT1 upon IL-6 stimulation (Fig. 8, lane 1). This response can be enhanced by the addition of sIL-6R (Fig. 8, lane 2). As observed on HepG2 cells, sgp130 has only a weak influence on the IL-6 response (Fig. 8, lane 3). Again, the combination of sIL-6R and sgp130 led to a drastic reduction of the IL-6 response (Fig. 8, lane 4), confirming the antagonistic activity of sIL-6R in the presence of sgp130. Taken together, these findings allow some important conclusions on the physiologic role of the naturally occurring sIL-6R and sgp130 in human plasma, as will be outlined in the discussion.

Discussion

The analysis of complexes formed by iodinated rIL-6 in human serum by gel filtration revealed that a considerable amount of IL-6 is found in high molecular mass complexes of 450–100 kDa. sgp130 is also detected in the corresponding fractions, and its immunoprecipitation leads to the coprecipitation of iodinated IL-6. As previously shown by Narazaki et al. (20), by direct precipitation of serum samples and subsequent analysis by SDS-PAGE, we confirm the sIL-6R-dependent association of sgp130 with IL-6 in human serum. The peaks observed in gel filtration were relatively
broad, which may have been caused by protein-protein interactions of unknown specificity due to the high protein concentration in human plasma. Therefore, a clear separation of free sgp130 and sgp130 complexed with IL-6/sIL-6R was not achieved. Since the applied IL-6 amounts were relatively low, a clear shift of the sgp130 peak due to complex formation was not expected. The nature of the IL-6 peak eluting with the void volume of the column, which was more pronounced in the absence of sIL-6R, did not show any sgp130 immunoreactivity in ELISA. Interestingly, May et al. (32) have described the occurrence of large amounts of endogenous IL-6 in human blood in two high molecular mass complexes. They described the IL-6 peak corresponding to the IL-6/sIL-6R/sgp130 complexes we observed as biologically inactive (33). This is in line with our arguments that IL-6 is partially trapped and inactivated in a ternary complex with sIL-6R and sgp130.

After enrichment by immunoadfinity chromatography, sgp130 appears as a monomeric protein of 100 kDa. Whether sgp130 is generated by shedding of the membrane-bound receptor or by translation of an alternatively spliced mRNA is not clear. Phorbol ester induces shedding of the IL-6R (34). Shedding of gp130, however, could hardly be detected (35). While the sIL-6R generated by alternative splicing (36) has been identified in human plasma (26), detection of the protein encoded by the alternatively spliced gp130 mRNA (37) has not been reported so far.

To investigate its antagonistic activity, in vitro studies were performed with rsgp130 from baculovirus-infected insect cells. rsgp130 was purified to homogeneity in a single step by immunoadfinity chromatography. Compared with sgp130 from human plasma, the purified rsgp130 showed a markedly lower apparent molecular mass as determined by SDS-PAGE, as well as by gel exclusion chromatography. The difference in molecular mass is most probably due to a different degree of glycosylation. A similar discrepancy in glycosylation levels was observed for the sIL-6R from human plasma (70 kDa) (26) and recombinant sIL-6R from baculovirus-infected insect cells (45 kDa) (24). Since purified rsgp130 has a tendency to aggregate upon aging, the carbohydrate moiety of the soluble receptor from human blood may be required to stabilize the protein.

Maximal proliferation of our stably transfected BaF3-gp130 cells was achieved with 10 ng/ml (0.5 nM) IL-6 and 1 µg/ml (20 nM) sIL-6R. For half-maximal inhibition of the proliferative response (ID₅₀), 100 ng/ml (1.4 nM) rsgp130 was sufficient (see Fig. 5). IL-6/sIL-6R-induced proliferation was completely blocked at a concentration of 1 µg/ml (14 nM) rsgp130. This is a 28-fold excess over the IL-6 concentration used in this experiment. The same concentration of rsgp130 was sufficient to inhibit the rapid IL-6/sIL-6R-induced activation of STAT1 in COS-7 cells (Fig. 6). Presumably, rsgp130 is such a potent antagonist because it neutralizes IL-6/sIL-6R complexes by forming high affinity ternary complexes. In previous studies, IL-6 and sIL-6R variants were designed to neutralize IL-6 via the low affinity IL-6/sIL-6R interaction. A concentration of at least 100 nM sIL-6R mutated in the predicted IL-6R/sgp130 interface was required to achieve a partial inhibition of the IL-6 response (38). IL-6 mutated in the predicted gp130-binding sites must be applied at a 1000-fold or larger excess to efficiently inhibit IL-6 responses (39, 40). The fact that the antagonistic activity of IL-6 variants was enhanced by additional mutations strengthening the IL-6/sIL-6R interaction is in line with the above arguments (40).

On cells expressing membrane-bound IL-6 receptor that were stimulated with IL-6 alone, we found that rsgp130 was a much weaker antagonist. A concentration of 1 µg/ml rsgp130 was insufficient both on HepG2 and on MDCK/IL-6R cells to completely inhibit the IL-6 response, although a significant reduction was observed in both cases. Addition of sIL-6R, which in the absence of

FIGURE 9. Concentration of bioactive IL-6 in the presence of 300 ng/ml (3000 pM) sgp130 and 50 ng/ml (700 pM) sIL-6R as a function of the initial IL-6 concentration. Given the physiologic (initial) concentration of sIL-6R ([sIL-6R]₀ = 0.7 nM) and the dissociation constant of the IL-6/sIL-6R interaction (K_D₁, IL-6 + sIL-6R ≈ IL-6/sIL-6R), the concentration of IL-6 bound to sIL-6R can be calculated by solving Equation 1,

\[ K_D₁ = \frac{[sIL-6R]₀ - [IL-6/sIL-6R]/[IL-6] - [IL-6/sIL-6R]}{[IL-6]/sIL-6R]₀} \]  

which results in Equation 2

\[ [IL-6]/sIL-6R]₀ = 0.5[sIL-6R]₀ + 0.5[IL-6] + 0.5K_D₁ - 0.5([sIL-6R]₀ - 2[sIL-6R]/[IL-6] + 2[IL-6]/K_D₁) + 0.5 \]  

Assuming that binding of one gp130 molecule is sufficient to inactivate the IL-6/sIL-6R complex, the portion of IL-6 antagonized because of ternary complex formation can be approximately calculated by solving Equation 3, in which K_D₂ corresponds to the dissociation constant of the interaction of sgp130 with IL-6/sIL-6R (K_D₂ = IL-6/sIL-6R + sgp130 ≈ IL-6/sIL-6R/sgp130).

\[ K_D₂ = \frac{[IL-6]/sIL-6R]₀ - [IL-6]/sIL-6R]/[sgp130])_{[sgp130]} - [IL-6]/sIL-6R/sgp130)}{[IL-6]/sIL-6R/sgp130]₀} \]  

To solve Equation 3 to calculate the concentration of the ternary complex, the initial IL-6/sIL-6R concentration ([IL-6]/sIL-6R) was replaced by Equation 2. The initial concentration of sgp130 ([sgp130]) is the one measured in human blood (3 nM). Using the solved Equation 3, the concentration of ternary complexes was calculated as a function of the initial IL-6 concentration ([IL-6]). Subtraction of the concentration of IL-6 inactivated because of ternary complex formation from the initial IL-6 concentration results in the amount of bioactive IL-6. Dissociation constants used were the following: K_D₁, 5000 pM; and K_D₂, 500 nM. The dashed line marks the concentration of bioactive IL-6 in the absence of the soluble receptors ([bioactive IL-6] = [IL-6]); 100 pM IL-6 corresponds to 2 ng/ml.
sgp130 acts as an IL-6 agonist, now potentiates the antagonistic activity of sgp130. We provide a simple explanation for this phenomenon. In cases in which HepG2 or MDCK/IL-6R cells are stimulated with IL-6 alone, the cytokine first binds to the cell surface IL-6R before it interacts either with sgp130 or with membrane-bound gp130. Here, 1 &mu;l/ml sgp130 is not sufficient to strongly inhibit the IL-6 response, because it has to compete with membrane-bound gp130 present in a high local concentration. Moreover, the IL-6/IL-6R complexes, due to their membrane location, have to find membrane-bound gp130 only in a two-dimensional space. When sIL-6R is added, IL-6/sIL-6R complexes can be trapped by sgp130 in the soluble high affinity ternary complexes and are thereby efficiently neutralized before they bind to the cell surface receptors. Narazaki et al. (20) observed an only 50% reduction in IL-6 responses, even at an sgp130 concentration of 2 &mu;g/ml. Possibly, this weak inhibition is due to the low sIL-6R concentration (75 ng/ml) that these authors have used in their experiments.

What is the functional role of the naturally occurring sgp130 and sIL-6R? Since the concentration of sgp130 in human plasma (about 300 ng/ml or 3 nM; Ref. 20 and our data) is considerably higher than that of sIL-6R (about 50 ng/ml or 0.7 nM; Refs. 21 and 26), it is reasonable to assume that this soluble receptor pair has evolved to inhibit systemic IL-6 responses. Indeed, when HepG2 cells were stimulated with IL-6 in the presence of sIL-6R and rsgp130 at physiologic concentrations, sIL-6R enhanced the antagonistic effect of rsgp130 (Fig. 7). However, the effect was more pronounced at higher concentrations of IL-6, sIL-6R, and sgp130. To assess the functional role of the soluble receptor proteins more quantitatively, we performed some simple calculations applying the law of mass action (detailed in the legend to Fig. 9). In Fig. 9, the outcome is depicted as a series of graphs showing the concentration of bioactive IL-6 (IL-6), [IL-6/sIL-6R/sgp130] as a function of the initial IL-6 concentration (IL-6i) in the presence of physiologic concentrations of sgp130 and sIL-6R. If K313 has a value of 5000 pM (Fig. 9, circles) or higher, the bioavailability of circulating IL-6 is not influenced by sIL-6R and sgp130. At a K313 of 500 pM (Fig. 9, triangles), the concentration of bioactive IL-6 is considerably reduced to about 50% due to ternary complex formation unless the IL-6 concentration is in the range of or even exceeds the sIL-6R concentration. A K3 of 50 pM (squares) would lead to a very strong inhibition of IL-6 responses, since more than 90% of IL-6 is trapped in ternary complexes at moderately elevated IL-6 concentrations. Using recombinant sIL-6R from baculovirus-infected insect cells, we measured a K3 of 500 pM for the binding of iodinated IL-6 (24). A 10-fold higher affinity was measured for ternary complex formation on cells expressing IL-6R and gp130 (K3 = 50 pM (41, 42). Applying these dissociation constants to our mathematical model (Fig. 9, triangles) suggests that in the presence of sIL-6R and sgp130 at physiologic concentrations the systemic IL-6 response is modulated in such a way that the bioavailability of IL-6 decreases due to soluble ternary complex formation. Instead of being an agonist (19), sIL-6R of human plasma should therefore be regarded as a protein that enables sgp130 to efficiently trap IL-6 in a soluble ternary complex, thereby acting as a buffer to modulate systemic IL-6 responses. Furthermore, using the above dissociation constants, Equation 3 predicts that at the sgp130 and sIL-6R concentrations used in most of our experiments (1 &mu;g/ml each), the amount of bioavailable IL-6 is reduced 50-fold. This corresponds to the observed strong antagonistic effects. It should be taken into consideration that due to cell activation or certain pathologic conditions, the local concentrations of sgp130 and sIL-6R may be consider-

ably different from the ones observed in human plasma, thus modulating the inhibitory capacity of this pair of soluble receptors.

Modulation of biologic responses by antagonizing proteins is a common principle in cytokine biology. For example, the bioactivity of IL-1 can be down-regulated by a naturally occurring IL-1 receptor antagonist (43). For other soluble cytokine receptors, it has been demonstrated that they indeed play a physiologic role in the down-regulation of the response to the corresponding mediator. Very recently, a circulating leptin-binding protein has been detected in mice that is up-regulated during pregnancy. This protein efficiently neutralizes leptin and was identified as a soluble form of the leptin receptor (44). A similar functional role during pregnancy has been assigned to the soluble growth hormone receptor (45). Both receptors belong to the same family of cytokine receptors as do IL-6R and gp130. Also, for other members of the cytokine receptor family, soluble forms have been described (reviewed in Ref. 46). In each case, their physiologic roles need to be elucidated.

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References


