Interleukin (IL)-6 is involved in the maintenance and progression of several diseases such as multiple myeloma, rheumatoid arthritis, or osteoporosis. The present work aims at the development of an IL-6 inhibitor for the use in anti-cytokine therapies. The IL-6 receptor is composed of two different subunits, an α-subunit (IL-6Rα) that binds IL-6 with low affinity and a β-subunit (gp130) that binds the IL-6:IL-6Rα complex with high affinity and as a result triggers intracellular signaling. In its soluble form, gp130 is a natural antagonist that neutralizes IL-6-soluble IL-6Rα complexes. It was our strategy to appropriately fuse the two receptor subunit fragments involved in IL-6 receptor complex formation to bind IL-6 with high affinity and to antagonize its effects. The ligand-binding domains of gp130 (D1–D2–D3) and IL-6Rα (D2–D3) were connected using three different linkers. The resulting constructs were expressed in stably transfected insect cells and tested for their ability to inhibit IL-6 activity in several in vitro systems. All fusion proteins were strong inhibitors of IL-6 signaling and abrogated IL-6-induced phosphorylation of STAT3, proliferation of transfected Ba/F3 cells, and induction of acute-phase protein synthesis. As intended, the fused receptors were much more effective than the separately expressed soluble receptor proteins. The fusion protein strategy presented here can also be applied to other cytokines that signal via receptors composed of two different subunits to design new potent inhibitors for anti-cytokine therapies.

Anti-cytokine therapies are aimed at the inhibition of a certain cytokine that is responsible for the maintenance of a disease. Different strategies have been used to neutralize cytokines in patients. Most effective has been the application of soluble cytokine receptors that consist solely of the ectodomain but lack the transmembrane and cytoplasmic regions. They bind the respective cytokine with high affinity and specificity as membrane-bound receptors do. In the treatment of chronic inflammatory diseases such as rheumatoid arthritis, the use of dimeric soluble tumor necrosis factor receptors for the neutralization of tumor necrosis factor has been a real breakthrough (1).

IL-6 is secreted by several cell types in response to various inflammatory stimuli. It is the major mediator of the acute-phase response of the liver and is involved in the coordination of inflammatory and immune responses at the site of inflammation (2). In several acute and chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases, in postmenopausal osteoporosis, but also in certain types of cancer, IL-6 levels are elevated and a causal role for IL-6 in disease progression has been suggested. In some cases inhibition of IL-6 activity by receptor antagonists or neutralizing antibodies has beneficial effects (3, 4).

IL-6 belongs to the family of hematopoietic cytokines (5). It is a member of the subfamily of IL-6-type cytokines (6) comprising IL-6, IL-11, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, cardiotrophin-1, and cardiotrophin-like cytokine. They all use the hematopoietic cytokine receptor gp130 as a common signal-transducing receptor subunit (7). As a result of receptor activation the transcription factor STAT3 becomes tyrosine-phosphorylated and translocates into the nucleus to induce target gene expression (8, 9).

Expression of gp130 is not sufficient for cells to become responsive to IL-6. They additionally have to express the cytokine-specific α-receptor subunit IL-6Rα. This α-receptor is not involved in the initiation of the cytoplasmic signal transduction cascades but is essential for cytokine binding. Thus, activation of the receptor by IL-6 requires two steps: (i) low affinity IL-6 binding to IL-6Rα and (ii) subsequent recruitment of the complex of IL-6 and IL-6Rα to two gp130 molecules leading to the formation of a high affinity ternary complex (10).

Cells lacking IL-6Rα can be stimulated with the combination of IL-6 and soluble IL-6Rα (sIL-6Rα) (10). In such a situation, IL-6 binds to sIL-6Rα in solution and the heterodimer of IL-6/sIL-6Rα activates membrane-bound gp130. Soluble gp130 (sgp130) alone acts as a relatively weak IL-6 antagonist (11). Most interestingly, the antagonizing activity of sgp130 is substantially increased by the presence of sIL-6Rα (12). Both sIL-6R (13, 14) and sgp130 (11, 12) are found in high concentrations in human blood (about 50 and 300 ng/ml, respectively). This pair of soluble receptors might act as a natural IL-6 inhibitor to limit systemic IL-6 responses (12).

Structurally, IL-6 belongs to the family of the α-helix-bundle cytokines. IL-6Rα as well as gp130 belong to the family of class I cytokine receptors (5). The extracellular regions of IL-6Rα and gp130 consist of three (D1–D3) (15) or six domains (D1–D6) (16), respectively. D2 and D3 of IL-6Rα are involved in IL-6

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binding (17). The complex of IL-6 and IL-6Ra is bound by D1–D3 of gp130 (18). IL-6 contains three receptor-binding sites. Site I is occupied by D2-D3 of IL-6Ra, and sites II and III bind to D2-D3 and D1 of gp130, respectively (19–21). Based on the mutagenesis data and the recently solved structure of D1–D3 of gp130 bound to viral IL-6, which binds gp130 in the absence of any a- receptor, a reliable model of the IL-6–IL-6R ternary complex has been proposed (22).

Inhibition of IL-6 activity by the DNA of soluble receptors is challenging because of the bipartite nature of the IL-6 receptor. IL-6 alone does not bind to gp130. To be neutralized by sgp130, IL-6 must first bind to sIL-6Ra. A fusion protein of gp130 and sIL-6Ra would therefore guarantee that the agonistic complex of IL-6/sIL-6Ra is immediately neutralized. Only recently, due to the new structural data on the IL-6-receptor complex (22), a promising rational approach on how to design an IL-6-antagonist based on a fusion of sgp130 with sIL-6Ra became possible. In this study, we present a highly potent IL-6 antagonist consisting of the ligand-binding moieties of sgp130 and sIL-6Ra.

**EXPERIMENTAL PROCEDURES**

Cloning of the Fusion Proteins—A fragment corresponding to D2-D3 of IL-6Ra (Val310–Lys336) was amplified by PCR introducing a multiple cloning site (Smal, NotI, MluI, NheI) with the sense primer and a ApoI site, a stop codon instead of Met331, and a D3 (Met1–Pro336) with the same enzymes. Then three different linkers were added after digestion of the obtained chimeric construct with MluI (Promega, Madison, WI) and NheI (MBI Fermentas GmbH, St. Leon-Rot, Germany) and cloned into pSVL-gp130 (D1–D3) with the sense primer and a BamHI site with the anti-sense primer. The product was cut with Smal (Roche Diagnostic GmbH, Mannheim, Germany) and BamHI (MBI Fermentas GmbH, St. Leon-Rot, Germany) and cloned into pSVL-gp130 (D1–D3) (Met1–Pro336) digested with the same enzymes. Then three different linkers were added after digestion of the obtained chimeric construct with MluI (Promega, Madison, WI) and NheI (MBI Fermentas GmbH). The first linker (stalk-49) corresponding to the short extracellular membrane proximal part of IL-6Ra (Ala323–Val362) was produced by PCR. Its amino acid sequence is GSAATRAEN EVSTPMQALT TNKDDDNILF.

Expressed in Insect Cells—High 5 (H5) cells cultured in SF-9002 medium (Invitrogen, Paisley, Scotland) were stably transfected with the empty pBlues vector or vectors containing the fusion protein constructs, using the CellFECTIN method (Invitrogen). Cell supernatants were harvested every 3 days, cleared by centrifugation, and stored at −20 °C until use.

Protein Precipitation—The fusion proteins from cell supernatants were precipitated overnight at 4 °C with IL-6 covalently linked to CNBr-Sepharose (Amersham Biosciences AB, Uppsala, Sweden).

Purification of Fusion Proteins—200–500 ml of H5 cell supernatants containing the respective fusion proteins were applied to an IL-6-Sepharose column (2 ml) at 4 °C. After rinsing with phosphate-buffered saline, proteins were eluted with 6 ml of 2 M MgCl2. The eluate was dialyzed against phosphate-buffered saline or cell culture medium, and subsequently the amounts of fusion proteins were measured by ELISA.

Quantification of Fusion Proteins by ELISA—An ELISA procedure was performed as described previously (12), using 0.3 μg/well of FLAG monoclonal antibody (Sigma) for coating and 50 ng/well of biotinylated monoclonal antibody B-T2 (DIACLINE, Besançon, France) as secondary antibody. The standard curve was obtained by 2-fold serial dilutions of sgp130-FLAG expressed in COS-7 cells and calibrated by sgp130 ELISA (12).

RESULTS

Rational Design of a Fusion Protein of sgp130 and sIL-6Ra as a Potential IL-6 Inhibitor—The fusion protein was designed to contain the minimal regions of IL-6Ra and gp130 required for high affinity IL-6 binding. Moreover, the N terminus of mature gp130 should not be affected by the fusion, since it is important for ligand binding (24). Thus, the fusion protein consists of domains D1–D2–D3 of gp130 (including the signal sequence at the N terminus that directs its secretion) followed by a linker and domains D2 and D3 of IL-6Ra (Fig. 1A, upper scheme). The two receptor fragments have to be connected by the linker in a way that allows the fusion protein to adopt the conformation required for efficient neutralization of IL-6. According to the ternary complex model based on the x-ray structure of viral IL-6 bound to D1–D3 of gp130 (22), the C terminus of gp130–D3 and the N terminus of sIL-6Ra-D2 are separated by at least 5 nm. This distance can be bridged by a peptide linker of about 30–40 amino acids (Fig. 1A, lower part). The linker should be of high conformational flexibility, of low immunogenicity, and resistant to protease degradation. Three fusion proteins containing different linkers were constructed. Two of them, AGS-33 and AGS-41, are made of flexible Ala-, Gly-, and Ser-rich peptides of 33 and 41 amino acids, respectively. In an extended conformation these linkers span from about 10 (AGS-33) to 12 nm (AGS-41). The third one (fp stalk-49) consists of a short flexible fragment of the extracellular membrane-proximal part of IL-6Ra (25). Besides its flexibility, this linker is expected to be of low immunogenicity, since it is derived from the endogenous IL-6Ra. For technical reasons, a FLAG tag epitope was added at the C termini of all constructs (Fig. 1A, upper scheme).

Purification and Characterization of Fusion Proteins Produced in Insect Cells—For continuous production of the fusion proteins stably transfected H5 insect cell lines were generated. The fusion proteins were precipitated from cell supernatants with IL-6-Sepharose and analyzed by Western blotting (Fig. 1B). The apparent molecular masses of the fusion proteins are 83.5 kDa for fp stalk-49, 69.5 kDa for fp AGS-33, and 72 kDa for fp AGS-41. The substantially higher molecular mass of fp stalk-49 is most likely due to an additional N-glycosylation site (Asn-Ala-Thr) introduced with the linker.

We took advantage of the affinity of the fusion proteins to IL-6 for their purification and concentration with IL-6-Sepharose. The insect cell supernatant, the flow-through and the eluate of IL-6 affinity chromatography were analyzed for the presence of fusion protein by Western blotting (Fig. 1C). Compared with the supernatant (un, left lane), the fusion protein is strongly enriched in the eluate. No fusion protein is detectable in the flow-through fractions. The concentrations of fusion protein in the fractions determined by a newly developed ELISA correlate well with the intensities of the bands in the Western blot (Fig. 1C). After dialysis, enriched fp stalk-49 was used for the following studies. Supernatants containing the other fusion
proteins and supernatants of mock-transfected insect cells were treated the same way. The latter was used as negative control in the bioassays.

**Potent IL-6 Antagonistic Activity of the Fusion Proteins**—To test the IL-6 antagonizing activity of the fusion proteins, supernatants of stably transfected insect cells were incubated with IL-6 for 30 min to allow the fusion protein to bind to IL-6. Ba/F3 cells stably transfected with gp130 and IL-6Ra (Ba/F3-gp130-IL6R) were stimulated with the IL-6-treated supernatants. After 30 min, cells were lysed, and STAT3 phosphorylation was analyzed. In the presence of supernatant from mock-transfected insect cells, stimulation of Ba/F3-gp130-IL6R cells with 0.5 ng/ml IL-6 is sufficient to induce prominent tyrosine phosphorylation of STAT3 (Fig. 2A, upper panel, lanes 1 and 2). Treatment of Ba/F3-gp130-IL6R cells with IL-6 that was preincubated with supernatants of cells expressing the fusion proteins did not result in significant tyrosine phosphorylation of STAT3 (Fig. 2A, upper panel, lanes 3–5). Thus, all three fusion proteins in the supernatants inhibit IL-6 signaling, since no STAT3 phosphorylation is observed in response to IL-6. A 2-fold higher IL-6 concentration (1 ng/ml) is neutralized only incompletely by the supernatant containing gp130-sIL-6Ra (Fig. 2A, lower panel, lane 4).

IL-6 is the major inducer of acute-phase protein synthesis in hepatocytes, but also in hepatoma cell lines such as HepG2. IL-6 stimulation (1 ng/ml) leads to a substantially increased α1-anti-chymotrypsin production by HepG2 cells as shown by immunoprecipitation of metabolically labeled protein (Fig. 2B, lanes 1 and 2). Purified proteins of control supernatant do not affect α1-anti-chymotrypsin synthesis (Fig. 2B, lane 3). In the presence of the concentrated fusion proteins (30 ng/ml), α1-anti-chymotrypsin synthesis is reduced to the basal level (Fig. 2B, lanes 4–6). Thus, all three fusion proteins inhibit IL-6-induced acute-phase protein synthesis.

**Specificity of the IL-6-inhibiting gp130-sIL-6Ra Fusion Proteins**—To demonstrate the specificity of the inhibitory fusion proteins, supernatants from mock-transfected insect cells expressing fusion proteins or from mock-transfected insect cells (control) and stimulated with 0.5 ng/ml IL-6 were incubated with supernatants from mock-transfected insect cells expressing gp130-IL6R or mock-transfected insect cells (control) and stimulated with different concentrations of IL-6 for 30 min as indicated. Cellular lysates were analyzed for STAT3 phosphorylation as described above. HepG2 cells were incubated with 1 ng/ml IL-6 and 30 ng/ml of fusion-proteins or an equivalent volume of purified mock-vector-transfected cell supernatant (control) for 18 h and metabolically pulse-labeled with 35S for 3 h. Secreted α1-anti-chymotrypsin (α1-ACT) was immunoprecipitated from cell culture supernatants, separated by SDS-PAGE, and analyzed by autoradiography.
proteins, we compared the proliferation of Ba/F3 cells stably transfected with gp130 and IL-6Ra or gp130 and IL-11Ra (Ba/F3-gp130-IL11R) in response to 0.9 ng/ml IL-6 or 5 ng/ml IL-11, required for 50 or 80% of maximal cell proliferation, respectively. Trx-IL-11 is a fusion protein of thioredoxin and IL-11, required for 50 or 80% of maximal cell proliferation, respectively. IL-6Rα-receptor binding. Although the superantagonists perform better, they still have to be applied in a large excess to IL-6 (27). Furthermore, due to the many mutations the proteins are highly immunogenic (28). Neutralizing IL-6 or IL-6Ra antibodies have also been used as IL-6 inhibitors. They were tested in clinical trials for the treatment of rheumatoid arthritis (3) or AIDS-associated Kaposi's sarcoma (29) but turned out to be of rather low efficiency. Very recently, potent low molecular mass IL-6 receptor antagonists were described for the first time (30, 31). These antagonists have to be applied in the micromolar range to inhibit picomolar amounts of IL-6.

A new generation of cytokine antagonists is based on soluble receptor fragments that bind the ligand with high affinity and specificity. In the case of IL-6, two receptor subunits are required for high affinity binding: IL-6Ra and gp130. Moreover, the complex of IL-6 and sIL-6Ra acts agonistically on cells expressing gp130 (10). Conversely, sIL-6Ra supports neutralization of IL-6 by sgp130 due to formation of a soluble high affinity ternary complex (12). The new IL-6 receptor antagonist presented in this study stems from the idea that appropriate fusion of the ligand-binding domains of IL-6Ra and gp130 should result in a superior antagonist that neutralizes IL-6 with highest affinity and specificity. In the present study three different linkers were used to connect the ligand-binding domains of gp130 and IL-6Ra. It turned out that the fusion proteins exhibit similar inhibitory activities, indicating that the estimation of the required linker length has been correct and appropriate peptide linkers were chosen.

All three fusion proteins bind IL-6 as shown by precipitation with IL-6-Sepharose. The fusion protein present in the insect cell supernatant is sufficient to completely antagonize the activity of 0.5 ng/ml (25 μg) IL-6 in the short term STAT3 phosphorylation assay using transfected Ba/F3 cells (Fig. 2A). Since the concentrations of the fusion proteins in the insect cell supernatants are in the range of 1–2 ng/ml (15–30 μg/ml), this points to an inhibitory activity at a molar ratio between agonist and antagonist of 1:1.

In a long term assay such as induction of acute-phase protein synthesis in HepG2 cells, the activity of 1 ng/ml (50 μl) IL-6 was totally blocked by the addition of inhibitory fusion protein at a nearly 10-fold molar excess (450 μl). In the Ba/F3 proliferation assay with fp stalk-49, we determined an IC50 of 6 ng/ml (90 μl) for the inhibition of 0.9 ng/ml IL-6 (45 μl). Thus, in long term assays and therefore also for studies of the inhibitory activity of the fusion proteins in vivo, an about 10-fold molar excess of fusion protein over IL-6 should be applied.

Besides their inhibitory activity the specificity of the fusion
proteins is an important feature to assess their potential value for anti-cytokine therapies. IL-11 most closely resembles IL-6 because it also signals via gp130 homodimers but binds to a different α-receptor, namely IL-11Rα. In the Ba/F3-proliferation assay, amounts of fusion proteins that significantly inhibit IL-6 activity had no effect on IL-11 induced proliferation (data shown only for fp stalk-49). Therefore, at the concentrations used in our assays each of the three fusion proteins is a potent and specific inhibitor of IL-6 activity.

The superior activity of the fused ligand-binding domains of gp130 and IL-6Ra compared with the separate soluble receptors sgp130 and sIL-6Rα is probably the most important issue left to be proven to confirm the value of our concept. IL-6-induced STAT3 phosphorylation in Ba/F3 cells is inhibited by the presence of equimolar amounts of fusion protein. To achieve a similar inhibition an at least 100-fold molar excess of the fusion proteins is an important feature to assess their potential value for the development of new anti-cytokine therapies.

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A Fusion Protein of the gp130 and Interleukin-6Rα Ligand-binding Domains Acts as a Potent Interleukin-6 Inhibitor
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