Novel Inhibitors for Murine and Human Leukemia Inhibitory Factor Based on Fused Soluble Receptors*

Silke Metz, Gudrun Naeth, Peter C. Heinrich, and Gerhard Müller-Newen

From the Institut für Biochemie, Universitätsklinikum RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Fusion proteins of the extracellular parts of cytokine receptors, also known as cytokine traps, turned out to be promising cytokine inhibitors useful in anti-cytokine therapies. Here we present newly designed cytokine traps for murine and human leukemia inhibitory factor (LIF) as prototypes for inhibitors targeting cytokines that signal through a heterodimer of two signaling receptors of the glycoprotein 130 (gp130) family. LIF signals through a receptor heterodimer of LIF receptor (LIFR) and gp130 and induces the tyrosine phosphorylation of STAT3 leading to target gene expression. The analysis of various receptor fusion and deletion constructs revealed that a truncated form of the murine LIF receptor consisting of the first five extracellular domains was a potent inhibitor for human LIF. For the efficient inhibition of murine LIF, the cytokine-binding module of murine gp130 had to be fused to the first five domains of murine LIFR generating mLIF-RFP (murine LIFR fusion protein). The tyrosine phosphorylation of STAT3 and subsequent gene induction induced by human or murine LIF are completely blocked by the respective inhibitor. Furthermore, both inhibitors are specific and do not alter the bioactivities of the closely related cytokines interleukin (IL)-6 and oncostatin M. The gained knowledge on the construction of LIF inhibitors can be transferred to the design of inhibitors for related cytokines such as IL-31, IL-27, and oncostatin M for the treatment of inflammatory and malignant diseases.

Proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), or interleukin-6 (IL-6) have been identified as promising therapeutic targets in the treatment of chronic inflammation. A dimeric soluble TNF receptor is currently used for the treatment of inflammatory diseases caused by elevated TNF expression (1). Whereas TNF signals through a receptor homotrimer, most cytokines signal through receptor complexes consisting of two or more different receptor subunits. In this case, the respective cytokine can be inhibited by using fusion proteins composed of the different soluble receptors, as we and others showed for the inhibition of IL-6 (2–4).

All cytokines signaling through the common receptor subunit gp130 belong to the family of IL-6-type cytokines (5), which includes IL-6, IL-11, IL-27, LIF, OSM, ciliary neurotrophic factor, cardiotoxin-1, cardiotoxin-like cytokine, and neuropoietin. IL-6-type cytokines contain distinct receptor-binding sites that were discovered by mutagenesis studies on IL-6, ciliary neurotrophic factor, and LIF (6–8). The IL-6-type cytokines can be subdivided into those containing three (I, II, and III) or two (II and III) receptor-binding sites. Site I determines the specificity of α-receptor binding. The α-receptor is not capable of transferring the signal into the cell but is crucial for increasing the binding affinity of the cytokine to its signaling receptors. Site II seems to be the universal gp130-binding site of all IL-6-type cytokines. Depending on the cytokine, site III is used for the recruitment of LIFR, OSMR, or a second gp130 molecule (5). The IL-6 inhibitor IL-6-RFP (2, 3) was designed to block a cytokine containing all three receptor-binding sites.

However, there are also IL-6-type cytokines, which do not need to recruit an α-receptor analogous to human IL-6Rα, and thus do not seem to have a functional site I. One example for a cytokine belonging to this group is the leukemia inhibitory factor (LIF). In this study we present an approach to construct inhibitory receptor fusion proteins for human and murine LIF as prototypes of inhibitors targeting cytokines whose receptors only bind to the site II and III of the cytokine without occupying site I. We designate these inhibitors “site II/III inhibitors.”

LIF signals through a heterodimer of LIFR and gp130. Janus kinase 2 (JAK2) is constitutively associated with the cytosolic parts of gp130 (9) and LIFR (10) and is activated upon ligand binding and phosphorylates the receptors and the recruited transcription factor STAT3. Activated STAT3 dimerizes and translocates into the nucleus, where it induces LIF target genes (11).

We wanted to integrate only those receptor domains of gp130 and LIFR into the inhibitory receptor fusion proteins that are necessary for LIF binding. For gp130, which includes six extracellular domains (D1–D6), it has been clearly shown that domains D2 and D3 forming the cytokine-binding module (CBM) are necessary and sufficient for LIF binding (12). In contrast, there are contradictory statements in the literature with regard to the domains of LIFR involved in LIF binding. The LIFR is a protein of 190 kDa, which includes eight extracellular domains (D1–D8); these are an N-terminal CBM (D1
and D2), an Ig-like domain (D3), a C-terminal CBM (D4 and D5), and three fibronectin-type III-like (FNIII) domains (D6–D8) (13) (see left scheme in Fig. 1A). The LIFR belongs to the family of all cytokine receptors. Within this family the three membrane-proximal FNIII domains are dispensable for ligand binding, as it was shown by mutagenesis studies of the granulocyte colony-stimulating factor receptor (14) and gp130 (15). In the literature, different parts of the LIFR are proposed as the minimal requirement for LIF binding. Apart from a few studies in which the binding between hLIF and mLIFR was investigated (16, 17), most studies focused on the binding of hLIF to hLIFR. Whereas two groups (17, 18) described that the domains D1–D5 of LIFR are needed to bind LIF, another group assumes (16, 17), most studies focused on the binding of hLIF to hLIFR.

In the literature, different parts of the LIFR are proposed as the minimal requirement for LIF binding. Apart from a few studies in which the binding between hLIF and mLIFR was investigated (16, 17), most studies focused on the binding of hLIF to hLIFR. Whereas two groups (17, 18) described that the domains D1–D5 of LIFR are needed to bind LIF, another group assumes (16, 17), most studies focused on the binding of hLIF to hLIFR.

In this study we describe the development of specific inhibitors for human and murine LIF based on the ligand-binding domain and the C-terminal CBM of LIFR. The N-terminal CBM does not bind LIF at all. The N-terminal CBM is required for ciliary neurotrophic factor binding and signaling (20, 21). In view of these hypotheses we fused the proposed domains of murine LIFR to the CBM (D2 and D3) of human or murine gp130 to generate putative human or murine LIF inhibitors, respectively.

It had already been demonstrated that the natural inhibitor of murine LIF, namely the LIF-binding protein (LBP), a truncated, extracellular form of the mLIFR, binds mLIF with relatively low affinity (about 600–2000 pM) (16, 17) similar to the membrane-bound mLIFR. It was our aim to increase the binding affinity of LBP to mLIF by fusing distinct domains of the mLIFR to the CBM of murine gp130 and thereby generating a high affinity inhibitor for murine LIF.

For the construction of potent human LIF inhibitors, we made use of the unusual species cross-reactivity of mLIFR toward human LIF. Although hLIF binds to the hLIFR with a relatively low affinity (about 600–2000 pM), it binds to the mLIFR with a much higher affinity (about 10–20 pM). This phenomenon is primarily mediated by the Ig-like domain (17). Therefore, we integrated domains of the mLIFR not only into the possible murine but also into the possible human LIF inhibitors. In this study we describe the development of specific inhibitors for human and murine LIF based on the ligand-binding domains of the corresponding soluble receptors.

**Experimental Procedures**

**Cloning of LIF Inhibitors—**All LIF inhibitors were constructed in the same way; the desired domains of mLIFR were located behind the N-terminal signal sequence and fused through a peptide linker to D2 and D3 of human or murine gp130. C-terminally a FLAG tag was added. Human LIF inhibitors contained the linker stalk-49 (2) and murine LIF inhibitors the linker AGS-41 (2) consisting of 49 and 41 amino acids, respectively. The domains of the murine LIFR were flanked by XbaI and Xmal restriction sites, the linkers by Xmal and Nhel, the respective gp130(D2–D3) fragment by Nhel and Apal, and the FLAG tag by Apal and BamHI restriction sites. All LIF-RFP constructs were cloned into the eukaryotic expression vector pSVL that contains an SV40 promoter (GE Healthcare). For the amplification of human gp130(D2–D3), the plasmid pSVL-hgp130 was used as a template, which contains the complete cDNA of human gp130. For generating constructs containing domains of murine LIFR or murine gp130, whole mRNA isolated from mouse liver was reversely transcribed into cDNA using random primers. The cDNA served as a template for amplifying the desired sequences of mLIFR or mgp130. For cloning of the construct mLIFR(D2–D4)-mgp130(D2–D3), the plasmid pSVL-mLIFR(D2–D4)-hgp130(D2–D3) was cut with Xmal and Ndel, and the released DNA insert was replaced by a fragment derived from pSVL-mLIF-RFP cut with the same enzymes. In the same fashion, mLIFR(D3–D5)-mgp130(D2–D3) was cloned using pSVL-mLIFR(D3–D5)-hgp130(D2–D3) and the restriction enzymes Xmal and Sall.

For the stable expression of mLIFR(D1–D5) and mLIF-RFP in Hek293 Flp-In T-Rex expression cell lines (see below), the constructs were subcloned into the expression vector pcDNA5/FRT/TO that contains a doxycycline-inducible cytomegalovirus promoter (Invitrogen).

Constructs and primers used for cloning are as follows: pSVL-mLIFR(D1–D3)-hgp130(D2–D3), primers 1–4; pSVL-mLIFR(D3-hgp130(D2–D3), primers 5–8; pSVL-mLIFR(D1–D5)-hgp130(D2–D3), primers 9 and 10; pSVL-mLIFR(D2–D4)-hgp130(D2–D3), primers 5, 6, 11, and 12; pSVL-mLIFR(D3–D5)-hgp130(D2–D3), primers 5, 6, 7, and 12; pSVL-mLIFR(D1–D5), primers 9 and 15; pSVL-mLIFR(D1–D3)-mgp130(D2–D3), primers 15 and 16; pSVL-mLIFR(D3–D5)-mgp130(D2–D3), primers 15 and 16; pSVL-mLIF-RFP = pSVL-mLIFR(D1–D5)-mgp130(D2–D3), primers 14 and 15; pcDNA5/FRT/TO-mLIF-RFP, primers 17 and 18. The primer sequences are as follows: 1) mLIFRXbaIs 5′-TCTAG ATATGG CAGCT TACTC ATGGT G-3′; 2) mLIFRXmaIas 5′-CGCTT TCCTC CAGAT AAACC-3′; 3) hgp130D2–D3Nhel 5′-GCTAG CTCAG GCTTG CCTCC AGAAA AACC-3′; 4) hgp130D2–D3Apalas 5′-GGGCC CTGGT CTATC TTCAT 5′; 5′-GCTAA GGAGC TCACC GTTTG CATGC ATCGT CAG-3′; 6) SIG(mLIFR)SacIas 5′-GCTAA GCTCG AGTCT AGAAAT GGCAG CTTAC 3′; 7) mLIFRD3SacIs 5′-TCTAG AAAGC TCGAG TCAG ACTAA TGT TT TT CTCAAGA C-3′; 8) hgp130D2as 5′-AGTTT TTTCT GTAGG CAGAG CTGA-3′; 9) mLIFDRD1–D5XmaIs 5′-GCTAA GAGAGC CACCT GCCAG ATCGT CAG-3′; 10) mLIRDR1–D5Xmalas 5′-CCTAA GGACG TCCGG ACTAA GGTGG CTATC ATCGT CAG-3′; 11) mLIRDR2–4Sacls 5′-GCTAA GAGAGC TCCCA GAGAC TCCGG ATCGT CAG-3′; 12) mLIRDR2–4Xmalas 5′-AGCTT CCTCGG CCTCGG ACTAA GGTGG CTATC ATCGT CAG-3′; 13) mLIFRD1–D5XmaIs 5′-GCTAA GAGAGC TCCGG ATCGT CAG-3′; 14) mLIRDR2–4Sacls 5′-GCTAA GAGAGC TCCCA GAGAC TCCGG ATCGT CAG-3′; 15) mLIRDR3–D2Apalas 5′-GCTAA GAGAGC TCCGG ATCGT CAG-3′; 16) mLIRDR3–D2Nhel 5′-GCTAA GAGAGC TCCGG ATCGT CAG-3′; 17) mLIFRFps 5′-GCTAA GAGAGC TCCGG ATCGT CAG-3′; 18) mLIRREpas 5′-GCTAA GAGAGC TCCGG ATCGT CAG-3′.
Cytokines, Cytokine Receptors—Human and murine LIF was purchased from Chemicon International (Temecula, CA) and murine OSM from R & D Systems (Minneapolis, MN). Recombinant human IL-6 was expressed in Escherichia coli, refolded, and purified as described (22). The specific activity of IL-6 was measured in the B9 cell proliferation assay (23). sIL-6Rα was expressed in insect cells and purified as described previously (24).

Cell Culture of MEF and COS-7 Cells and Transfection of COS-7 Cells—COS-7 simian monkey kidney cells (kindly provided by I. M. Kerr, Cancer Research UK, London) and murine embryonic fibroblasts (MEF) (kindly provided by B. Neel, Boston) were grown in Dulbecco's modified Eagle's medium with GlutaMAX™-I, 4.5 g/liter glucose and pyruvate (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Cytogen, Princeton, NJ), 100 μg/ml streptomycin, and 100 units/ml penicillin (Cambrex BioScience, Verviers, Belgium). Cells were grown at 37 °C in a water-saturated atmosphere in 5% CO₂. Plasmids were transiently transfected into COS-7 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

Cell Culture and Transfection of HepG2 Cells—HepG2 human hepatoma cells (purchased from ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium F-12 1:1 mix with GlutaMAX™-I (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were grown at 37 °C in a water-saturated atmosphere in 5% CO₂. Plasmids were transiently transfected into HepG2 cells using FuGENE6 (Roche Applied Science) according to the manufacturer's instructions.

Preparation of Cell Lysates, SDS-PAGE, Western Blotting, and Immunodetection—COS-7 cells were transiently transfected with expression plasmids (pSVL) coding for the respective LIF inhibitor and grown for 48 h to allow protein production. Subsequently cells were lysed with radioimmune precipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM NaF, 15% glycerol, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, 0.25 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 1 μg/ml leupeptin). The lysates were analyzed with SDS-PAGE, Western blotting, and immunodetection using a 1:1,000 diluted antibody directed against the FlAG tag (Sigma). After stripping the membrane in stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.7, 76 μl of β-mercaptoethanol per 10 ml) for 25 min at 70 °C, a second immunodetection was performed using a FLAG antibody.

Generation of Stable HEK293 Flp-In T-Rex Expression Cell Lines for the Production of LIF-RFPs—For production of the LIF inhibitors, the Flp-In T-Rex protein expression system (Invitrogen) was used. The generation of stable HEK293 Flp-In T-Rex expression cell lines for LIF inhibitors was performed according to the manufacturer's instructions. In brief, HEK293 Flp-In T-Rex host cells were cotransfected with 3.6 g of the plasmid pOG44 (Invitrogen) coding for the Flp-In recombinase and 0.4 μg of the plasmid pcDNA5/FRT/TO (Invitrogen) containing the coding sequence for the desired LIF inhibitor. Cotransfection was carried out by using FuGENE 6 according to the manufacturer's instructions. 48 h after transfection stably transfected cell were selected with 50–200 μg/ml hygromycin B (Perbio/HyClone, Logan, UT). Single clones were expanded and treated with 10 ng/ml doxycycline (Sigma) for 24 h without serum and hygromycin B to induce protein production. The supernatants were cleaned by centrifugation and sterile filtration, and the production of the respective LIF-RFP was checked by SDS-PAGE, Western blotting, and immunodetection using a FLAG antibody.

Concentration of LIF-RFPs—After doxycycline treatment, supernatants of the respective HEK293 Flp-In T-Rex expression cells were harvested, cleaned by centrifugation and sterile filtration, and concentrated about 20-fold using Vivaspin concentrators (Vivascience AG, Hannover, Germany). The same was done with supernatants from control HEK293 Flp-In T-Rex cells, which were stably transfected with an empty vector.

ELISA-based Binding Assay—ELISA plates with a polystyrene surface (Nunc) were coated with mLIF (50 ng/well) overnight. Blocking of free binding sites was carried out using PBS supplemented with 3% bovine serum albumin and 10% fetal calf serum for 30 min. After three washing steps with 250 μl of PBS containing 0.005% Tween 20, the wells were incubated with varying concentrations of the indicated FLAG-tagged LIF inhibitor or control supernatant and a FLAG antibody (20 ng/well, Sigma) for 3.5 h. The plates were washed three times with 250 μl of PBS/Tween and incubated with a horseradish peroxidase-conjugated secondary antibody (20 ng/well) for 30 min. After three washing steps the horseradish peroxidase-catalyzed color reaction was initiated using a 0.1 M sodium acetate solution, pH 5.5, containing 100 μg/ml tetramethylbenzidine and 0.003% H₂O₂. The reaction was stopped by the addition of 2 M H₂SO₄. The absorption was measured with an ELISA reader. All incubation steps were carried out at room temperature.

STAT3 Tyrosine Phosphorylation in MEF Cells—For investigation of STAT3 tyrosine phosphorylation, MEF cells were grown on 6-well plates (9.6 cm²/well) and stimulated for 20 min with hLIF, mLIF, hIL-6/shIL-6Ra, or mOSM or the combination of cytokine and LIF-RFP in the absence of serum. Subsequently, the cells were lysed with radioimmune precipitation assay lysis buffer (see above). The lysates were analyzed with SDS-PAGE, Western blotting, and immunodetection using an
**Novel Inhibitors for Murine and Human LIF**

**A** Schematic presentation of the potential domain structure of the LIFR (**left part**) and the composition of the potential LIF inhibitors for human (**upper part**) and murine (**lower part**) LIF. In the structure of the LIFR, thin lines represent cysteine bridges, and thick lines indicate a WS WS WS motif. In all constructs, the LIFR domains are derived from mouse (m). The gpl30 domains in the LIF-RFPs for LIF are of human (h) origin, and the gpl130 domains in the LIF-RFPs for mLIF are of murine origin. Human domains are presented in white and murine domains are drawn in gray. The small rectangles at the N termini of the LIF inhibitors represent the signal sequence of mLIF, and the small C-terminal rectangles indicate a FLAG tag. For the potential human LIF inhibitors, we chose the linker stalk-49, which was already used for the IL-6-RFP constructed in our group (2). This linker consists of a short flexible 49-amino acid fragment of the extracellular, membrane proximal part of human IL-6R (45). The potential murine LIF inhibitors contained the linker AGS-41 (2), which is composed of a flexible Ala-, Gly-, and Ser-rich peptide of 41 amino acids. The molecular (mol.) masses were calculated without consideration of glycosylation. *abbrev.* is abbreviation, *B*, expression of the potential human LIF inhibitors. COS-7 cells were transfected with 10 ng of pSVL containing the coding sequence for the indicated human LIF inhibitor or empty vector (mock). 48 h after transfection, the cells were lysed, and the lysates were analyzed by SDS-PAGE, Western blotting, and immunodetection using a FLAG antibody. *C*, expression of the potential murine LIF inhibitors. COS-7 cells were transfected with 10 ng of pSVL containing the coding sequence for the indicated murine LIF inhibitor or empty vector (mock). 48 h after transfection, the cells were lysed and the lysates were analyzed by SDS-PAGE, Western blotting, and immunodetection using a FLAG antibody.

| A | LIFR | potential inhibitors of hLIF | abbrev. | structure | calc. mol. mass |
|---|---|---|---|---|---|
| | | mLIFR(D1–D5) | hglp130(D2–D3) | m1LIFR(D1–D5) | hglp130 |
| | | mLIFR(D1–D3) | hglp130(D2–D3) | m1LIFR(D1–D3) | hglp130 |
| | | mLIFR(D2–49) | hglp130(D2–D3) | m1LIFR(D2–49) | hglp130 |
| | | mLIFR(D3–D5) | hglp130(D2–D3) | m1LIFR(D3–D5) | hglp130 |
| | | mLIFR(D1–D5) | hglp130 | m1LIFR(D1–D5) | hglp130 |

**B** Schematic presentation of the potential human and murine LIF inhibitors and their expression in COS-7 cells. *A*, schematic presentation of the domain structure of the LIFR (**left part**) and the composition of the potential LIF inhibitors for human (**upper part**) and murine (**lower part**) LIF. In the structure of the LIFR, thin lines represent cysteine bridges, and thick lines indicate a WS WS WS motif. In all constructs, the LIFR domains are derived from mouse (m). The gpl30 domains in the LIF-RFPs for LIF are of human (h) origin, and the gpl130 domains in the LIF-RFPs for mLIF are of murine origin. Human domains are presented in white and murine domains are drawn in gray. The small rectangles at the N termini of the LIF inhibitors represent the signal sequence of mLIF, and the small C-terminal rectangles indicate a FLAG tag. For the potential human LIF inhibitors, we chose the linker stalk-49, which was already used for the IL-6-RFP constructed in our group (2). This linker consists of a short flexible 49-amino acid fragment of the extracellular, membrane proximal part of human IL-6R (45). The potential murine LIF inhibitors contained the linker AGS-41 (2), which is composed of a flexible Ala-, Gly-, and Ser-rich peptide of 41 amino acids. The molecular (mol.) masses were calculated without consideration of glycosylation. *abbrev.* is abbreviation. *B*, expression of the potential human LIF inhibitors. COS-7 cells were transfected with 10 ng of pSVL containing the coding sequence for the indicated human LIF inhibitor or empty vector (mock). 48 h after transfection, the cells were lysed, and the lysates were analyzed by SDS-PAGE, Western blotting, and immunodetection using a FLAG antibody. *C*, expression of the potential murine LIF inhibitors. COS-7 cells were transfected with 10 ng of pSVL containing the coding sequence for the indicated murine LIF inhibitor or empty vector (mock). 48 h after transfection, the cells were lysed and the lysates were analyzed by SDS-PAGE, Western blotting, and immunodetection using a FLAG antibody.

---

**antibody directed against phosphotyrosine (705)-STAT3 (Cell Signaling, Danvers, MA) or STAT3 (H190, Santa Cruz Biotechnology, Santa Cruz, CA). Both antibodies were used in a 1:1,000 dilution.**

**Reporter Gene Assay in HepG2 Cells—**HepG2 cells were seeded onto 6-well plates (9.6 cm²/well) and transiently cotransfected with pGL3-αM-Luc (construct with luciferase gene regulated by the STAT3-responsive αM-macroglobulin promoter) and pCRTM3-lacZ (β-galactosidase construct with a constitutively active promoter; Amersham Biosciences). Cells were stimulated with either 5 ng/ml hLIF and an appropriate volume of control supernatant (see above) or the combination of hLIF and mLIFR(D1–D5) for 16 h at molar ratios indicated. Preparation of cellular lysates and luciferase measurements were carried out according to the instructions of the manufacturer (Promega, Madison, WI). The luciferase activity values were normalized to the transfection efficiency that was determined as β-galactosidase activity. The experiments were carried out in triplicate, and the mean values and standard deviations were calculated.

**SOCS3 mRNA Levels in MEF Cells—**MEF cells were seeded onto 6-well plates and stimulated with either 5 ng/ml mLIF and an appropriate volume of control supernatant or the combination of mLIF and mLIF-RFP for 30 min at the molar ratios indicated. Subsequently, mRNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). The SOCS3 and GAPDH mRNAs were amplified with the One-step RT-PCR kit (Qiagen) utilizing sequence-specific primers and analyzed with gel electrophoresis. Primers for RT-PCR are as follows: mouse SOCS3, 5’-GGGTT GCAA AAAAA GGAG-3’ and 5’-GTGTA GCGTC AAGAC CCAGT-3’; mouse GAPDH, 5’-ACCA CAGTCC ATGCG ATCAC-3’ and 5’-TCCAC CACCC TGTTG CTGTA-3’ (25).

**RESULTS**

**Design and Expression of Different Fusion Proteins of LIFR and gpl30 as Potential LIF Inhibitors—**The potential human and murine LIF inhibitors were designed to contain the minimal parts of LIFR and gpl30 required for high affinity LIF binding. Because the domains of LIFR necessary for LIF binding are controversially discussed, we constructed six possible inhibitors for human LIF containing the proposed domains of mLIF needed for LIF binding (Fig. 1A). The five receptor fusion constructs for the inhibition of hLIF contain the
Novel Inhibitors for Murine and Human LIF

MARCH 7, 2008 • VOLUME 283 • NUMBER 10

were analyzed by SDS-PAGE, Western blotting, and immunodetection using a FLAG antibody. All inhibitors were expressed and had the expected molecular masses. In general, inhibitors with low molecular masses were better expressed than those with higher molecular masses. Because all inhibitors are glycosylated, their calculated molecular masses differ from their apparent molecular masses determined by SDS-PAGE.

The Potential Human LIF Inhibitors mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5) Bind Human LIF Most Efficiently—We tested all potential LIF inhibitors for their capacity to bind human LIF in coimmunoprecipitation assays. Therefore, COS-7 cells were transfected with pSVL vector containing the coding sequence for the corresponding LIF inhibitor or empty vector. The FLAG-tagged LIF inhibitors were precipitated from cell supernatants with a FLAG antibody, and subsequently, the precipitated proteins were incubated with human LIF to allow coimmunoprecipitation. One representative coimmunoprecipitation for each potential human LIF inhibitor is shown, e.g. 1/14 means binding of LIF inhibitor to hLIF was detected in 1 of 14 experiments.

Comparison of the Binding Capacity of the Human LIF Inhibitors

As mLIFR(D1–D5) is much better expressed than mLIFR(D1–D3), we directly compared the human LIF binding capacities of mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5). As mLIFR(D1–D5) is much better expressed than mLIFR(D1–D5)-hgp130(D2–D3), we precipitated mLIFR(D1–D5) from decreasing volumes of supernatants of transfected COS-7 cells (Fig. 2B). When we used 0.75 ml of supernatant for precipitation of mLIFR(D1–D5) and 6 ml of supernatant for precipitation of mLIFR(D1–D5)-hgp130(D2–D3), the inhibitor amounts in the coimmunoprecipitations were comparable. Both

CBM (D2–D3) of human gp130, which was shown to be sufficient for LIF binding (12). To investigate the role of hgp130 in a human LIF inhibitor containing mLIFR, we constructed the LIF inhibitor mLIFR(D1–D5) consisting of D1–D5 of mLIFR, which was lacking the CBM (D2–D3) of human gp130 and the linker.

Accordingly, five potential murine LIF inhibitors were constructed by fusing the respective part of the mLIFR to the CBM of murine gp130. The two receptor fragments in the fusion proteins were connected by flexible linkers. For technical reasons, a FLAG tag was added to the C termini of all constructs.

The potential human (Fig. 1B) and murine (Fig. 1C) LIF inhibitors were expressed in COS-7 cells, the lysates of which

FIGURE 2. Coimmunoprecipitation of the potential human LIF inhibitors with human LIF. A, COS-7 cells were transfected with 10 μg of pSVL vector containing the coding sequence for the indicated potential human LIF inhibitor or empty vector. 48 h after transfection, the FLAG-tagged human LIF inhibitors were precipitated from cell supernatants with a FLAG antibody coupled to protein A-Sepharose. Subsequently, the precipitated proteins were incubated in PBS containing 50 nM human LIF to allow coimmunoprecipitation. The protein complexes were eluted from the protein A-Sepharose and investigated by SDS-PAGE, Western blotting, and immunodetection with a FLAG or hLIF antibody. Coimmunoprecipitation assays were performed several times; one representative coimmunoprecipitation for each potential human LIF inhibitor is shown. IP, immunoprecipitation; ab, antibody; PC, positive control. B, comparison of the binding capacity of the human LIF inhibitors mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5) to hLIF in coimmunoprecipitation assays carried out as described in A. To account for the strong expression of mLIFR(D1–D5), decreasing volumes of supernatants from COS-7 cells (COS-sn) expressing mLIFR(D1–D5) were used.

TABLE 1

Frequency of coimmunoprecipitation of the different proposed human LIF inhibitors with hLIF

Coimmunoprecipitation assays were performed several times, and the frequency of hLIF binding is shown, e.g. 1/14 means binding of LIF inhibitor to hLIF was detected in 1 of 14 experiments.

| Potential inhibitor for human LIF | Binding frequency of inhibitor to hLIF |
|----------------------------------|--------------------------------------|
| mLIFR(D1–D3)-hgp130(D2–D3)       | 1/4                                  |
| mLIFR(D3)-hgp130(D2–D3)          | 4/17                                 |
| mLIFR(D1–D5)-hgp130(D2–D3)       | 12/14                                |
| mLIFR(D2–D4)-hgp130(D2/D3)       | 1/10                                 |
| mLIFR(D3–D5)-hgp130(D2/D3)       | 1/6                                  |
| mLIFR(D1–D5)                      | 10/12                                |
| Mock                             | 1/22                                 |

human LIF inhibitor is presented in Fig. 2A. We found that mLIFR(D1–D3)-hgp130(D2–D3), mLIFR(D3)-hgp130(D2–D3), mLIFR(D2–D4)-hgp130(D2–D3), and mLIFR(D3–D5)-hgp130(D2–D3) bound human LIF only weakly, whereas mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5) coprecipitated with human LIF most efficiently. Because the results of the coimmunoprecipitation studies strongly depended on the transfection efficiency, we repeated them several times and determined the frequency of hLIF binding by the potential LIF inhibitors. As shown in Table 1, in most assays the truncated inhibitors failed to bind hLIF, whereas mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5) worked in almost each assay. Consequently, mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5) were chosen as suited human LIF-binding proteins.

D2 and D3 of hgp130 Are Dispensable for a Human LIF-binding Protein Based on mLIFR—To examine the role of the CBM (D2–D3) of hgp130 in the construct mLIFR(D1–D5)-hgp130(D2–D3), we directly compared the human LIF binding capacities of mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5). As mLIFR(D1–D5) is much better expressed than mLIFR(D1–D5)-hgp130(D2–D3), we precipitated mLIFR(D1–D5) from decreasing volumes of supernatants of transfected COS-7 cells (Fig. 2B). When we used 0.75 ml of supernatant for precipitation of mLIFR(D1–D5) and 6 ml of supernatant for precipitation of mLIFR(D1–D5)-hgp130(D2–D3), the inhibitor amounts in the coimmunoprecipitations were comparable. Both

Coimmunoprecipitation of the potential human LIF inhibitors with human LIF. A, COS-7 cells were transfected with 10 μg of pSVL vector containing the coding sequence for the indicated potential human LIF inhibitor or empty vector. 48 h after transfection, the FLAG-tagged human LIF inhibitors were precipitated from cell supernatants with a FLAG antibody coupled to protein A-Sepharose. Subsequently, the precipitated proteins were incubated in PBS containing 50 nM human LIF to allow coimmunoprecipitation. The protein complexes were eluted from the protein A-Sepharose and investigated by SDS-PAGE, Western blotting, and immunodetection with a FLAG or hLIF antibody. Coimmunoprecipitation assays were performed several times; one representative coimmunoprecipitation for each potential human LIF inhibitor is shown. IP, immunoprecipitation; ab, antibody; PC, positive control. B, comparison of the binding capacity of the human LIF inhibitors mLIFR(D1–D5)-hgp130(D2–D3) and mLIFR(D1–D5) to hLIF in coimmunoprecipitation assays carried out as described in A. To account for the strong expression of mLIFR(D1–D5), decreasing volumes of supernatants from COS-7 cells (COS-sn) expressing mLIFR(D1–D5) were used.

TABLE 1

Frequency of coimmunoprecipitation of the different proposed human LIF inhibitors with hLIF

Coimmunoprecipitation assays were performed several times, and the frequency of hLIF binding is shown, e.g. 1/14 means binding of LIF inhibitor to hLIF was detected in 1 of 14 experiments.

| Potential inhibitor for human LIF | Binding frequency of inhibitor to hLIF |
|----------------------------------|--------------------------------------|
| mLIFR(D1–D3)-hgp130(D2–D3)       | 1/4                                  |
| mLIFR(D3)-hgp130(D2–D3)          | 4/17                                 |
| mLIFR(D1–D5)-hgp130(D2–D3)       | 12/14                                |
| mLIFR(D2–D4)-hgp130(D2/D3)       | 1/10                                 |
| mLIFR(D3–D5)-hgp130(D2/D3)       | 1/6                                  |
| mLIFR(D1–D5)                      | 10/12                                |
| Mock                             | 1/22                                 |
mLIFR(D1–D5) and mLIFR(D1–D5)-hgp130(D2–D3) coprecipitated with human LIF to the same extent, indicating that D2 and D3 of hgp130 are dispensable for a human LIF inhibitor based on mLIFR.

**Fusion of D2 and D3 of mgp130 to mLIFR(D1–D5) Increases the Binding Affinity for Murine LIF**—It is known that the natural inhibitor of murine LIF, the LIF-binding protein (LBP) representing a truncated form of the cellular mLIFR, binds mLIF with a relatively low affinity (about 600–2000 pM) (16, 17). Because the human LIF inhibitor mLIFR(D1–D5) resembles the LBP, we asked whether mLIFR(D1–D5) also binds murine LIF. As hLIF binds to mLIFR with a much higher affinity than mLIF, we expected to find a binding affinity between mLIF and mLIFR(D1–D5) that is weaker than the binding affinity between hLIF and mLIFR(D1–D5).

As demonstrated in Fig. 3A, murine LIF is not coprecipitated at all with mLIFR(D1–D5) at the low LIF concentration (50 nM) we used in the assay. Even if large amounts of mLIFR(D1–D5) are precipitated, hardly any coprecipitation of mLIF is detectable (Fig. 3C). We next checked if mLIFR(D1–D5)-hgp130(D2–D3) containing human gp130(D2–D3) in addition to mLIFR(D1–D5) is capable of mLIF binding. We found that mLIFR(D1–D5)-hgp130(D2–D3) does not bind murine LIF at all (Fig. 3B), indicating that the binding affinity of hgp130 to mLIF is rather weak. We therefore constructed a new receptor fusion protein by replacing human gp130(D2–D3) in mLIFR(D1–D5)-hgp130(D2–D3) with murine gp130(D2–D3). mLIFR(D1–D5)-mgp130(D2–D3), abbreviated mLIF-RFP (murine LIF receptor fusion protein) strongly coprecipitates with murine LIF (Fig. 3C), showing that for efficient mLIF binding mLIFR(D1–D5) and mgp130(D2–D3) are needed and mLIF-RFP greatly exceeds the binding efficiency of the natural LIF inhibitor LBP to mLIF.

In an ELISA-based assay binding of mLIF-RFP and mLIFR(D1–D5),...
to immobilized mLIF was compared (Fig. 3D). A sigmoid saturation binding curve was observed for mLIF-RFP. With the concentrations applicable in the assay a saturation binding of mLIFR(D1–D5) could not be achieved. Nevertheless, from the shift of the binding curves it can be deduced that mLIF-RFP binds mLIF with at least 1 order of magnitude increased binding affinity compared with mLIFR(D1–D5).

The Domains D1 to D5 of mLIF Have to Be Integrated in the mLIF-RFP for Functional LIF Binding—Our next objective was to investigate whether it is possible to shorten the mLIF part in the mLIF-RFP consisting of the D1–D5 of murine LIFR fused to the D2 and D3 of murine gp130. Therefore, we cloned five potential murine fusion proteins that were truncated in the mLIFR part (see Fig. 1A, lower part). Those deletions were again chosen in relation to the proposed domains of LIFR needed for LIF binding. Resembling the results obtained for the potential human LIF receptor fusion proteins, none of the murine fusion proteins with truncations in the domains D1–D5 of mLIFR was useful as a potential mLIF inhibitor (Fig. 3E).

For mLIFR(D3)-mgp130(D2–D3), no secreted protein is detectable in the cell supernatant (Fig. 3E, lower panel). mLIFR(D3–D5)-mgp130(D2–D3) is only weakly secreted (Fig. 3E, middle panel). The other mutants are poor LIF binders. As a result, mLIF-RFP, containing D1–D5 of mLIFR fused with mgp130 (D2–D3), turned out to be the minimal high affinity mLIF inhibitor and was tested for its capacity to inhibit mLIF in cell culture experiments.

mLIFR(D1–D5) and mLIF-RFP Inhibit Tyrosine Phosphorylation of STAT3 after Stimulation with hLIF or mLIF, Respectively—For cell culture experiments larger amounts of both the inhibitor for human LIF (mLIFR(D1–D5)) and the inhibitor for murine LIF (mLIF-RFP) were needed. Therefore, we generated HEK293 Flp-In T-Rex cell lines stably expressing mLIFR(D1–D5) or mLIF-RFP after induction with doxycycline. An additional HEK293 Flp-In T-Rex expression cell line transfected with empty vector was generated to produce control supernatants. Lysates and supernatants of the stable cell lines were harvested, and cellular lysates were prepared. Additionally, mLIFR(D1–D5) (A) or mLIF-RFP (B) was precipitated from supernatants with a FLAG antibody previously coupled to protein A-Sepharose. Lysates (lys), supernatants (SN), or proteins precipitated from supernatants were analyzed by SDS-PAGE, Western blotting, and immunodetection with a FLAG or mLIF antibody. The expression of the respective inhibitor was quantified by comparing their signal intensities in an immunodetection with the signal intensities of the respective mLIFR (Fig. 4A, top panel). The medium-induced and noninduced cells stably transfected with the expression vector for mLIFR(D1–D5) are presented; B, protein expression profile of stably transfected cells with an expression vector for mLIF-RFP is shown. A, lysates of COS-7 cells transfected with mLIFR(D1–D5) were used as positive control (PC), hc, heavy chain.

Murine embryonic fibroblasts (MEF) are well suited to test both the inhibitor for human LIF (mLIFR(D1–D5)) and the inhibitor for murine LIF (mLIF-RFP), because they express mLIF on their cell surface, which responds to hLIF and mLIF. Stimulation of MEF cells with human or murine LIF leads to the tyrosine phosphorylation of STAT3. In Fig. 5, A and B, MEF cells were stimulated with 5 ng/ml hLIF or mLIF for 20 min in

FIGURE 3. Binding of mLIFR(D1–D5)-mgp130(D2–D3), mLIFR(D1–D5), and the potential murine LIF inhibitors to murine LIF. A, mLIFR(D1–D5) was precipitated from supernatants of COS-7 cells transfected with pSVL-mLIFR(D1–D5). The precipitated proteins were incubated in PBS containing 50 nm murine LIF to allow coimmunoprecipitation. The protein complexes were eluted from the protein A-Sepharose and analyzed by SDS-PAGE, Western blotting, and immunodetection with a FLAG or mLIF antibody. IP, immunoprecipitation; ab, antibody. PC, positive control. B, mLIFR(D1–D5)-mgp130(D2–D3) was precipitated from supernatants of COS-7 cells transfected with pSVL-mLIFR(D1–D5)-mgp130(D2–D3). The precipitated proteins were incubated in PBS containing 50 nm murine LIF to allow coimmunoprecipitation and analyzed as described in A. C, COS-7 cells were transfected with the pSVL vector containing the coding sequence for mLIF-RFP or mLIFR(D1–D5). Decreasing volumes of supernatants from COS-7 cells (COS-sn) expressing mLIFR(D1–D5) were used to precipitate different amounts of mLIFR(D1–D5). Afterward the precipitated proteins were incubated in PBS containing 50 nm murine LIF to allow coimmunoprecipitation and analyzed as described in A. D, ELISA-based binding assay. 96-Well plates were coated mLIF (50 ng/well) and incubated with the indicated concentrations of mLIF-RFP (solid circles, solid line), mLIFR(D1–D5) (open circles, solid line), or equivalent amounts of supernatant of mock-transfected cells (open triangles, dashed line). Bound inhibitors were detected with a FLAG antibody and a horseradish peroxidase-conjugated secondary antibody. E, COS-7 cells were transfected with the pSVL vector containing the coding sequence for the indicated potential murine LIF inhibitor (see also Fig. 1A). The recombinant proteins were precipitated from cell supernatants and incubated in PBS containing 50 nm murine LIF to allow coimmunoprecipitation. Afterward they were analyzed as described in A. The heavy chain band, which is detectable in the approaches without FLAG antibody (open triangles, dashed line), is probably derived from antibodies of the fetal calf serum in the culture medium.
Novel Inhibitors for Murine and Human LIF

**A**

![Image of STAT3 phosphorylation in MEF cells stimulated with hLIF, mLIF, mLIF-RFP, and mOSM and treated with mLIFR(D1–D5) or mLIF-RFP.](image)

**B**

![Image of STAT3 phosphorylation in MEF cells stimulated with mLIF-RFP and mLIF-RFP plus mLIFR(D1–D5).](image)

**C**

![Image of STAT3 phosphorylation in MEF cells stimulated with mLIF-RFP and mOSM.](image)

**D**

![Image of STAT3 phosphorylation in MEF cells stimulated with mLIF-RFP and mOSM plus mLIFR(D1–D5).](image)

**FIGURE 5.** STAT3 tyrosine phosphorylation in MEF cells stimulated with hLIF, mLIF, mLIF-RFP, and mOSM and treated with mLIFR(D1–D5) or mLIF-RFP. MEF cells were treated for 20 min at 37 °C with 5 ng/ml hLIF or mLIF in the presence or absence of mLIFR(D1–D5) or mLIF-RFP in the molar ratios indicated. Analysis of cross-reactivity of mLIFR(D1–D5) and mLIF-RFP toward mLIF. MEF cells were stimulated for 20 min at 37 °C with 5 ng/ml hLIF or mLIF in the presence or absence of mLIFR(D1–D5) at the molar ratios indicated. Afterward, cell lysates were analyzed as described above. D, investigation of the specificity of mLIF-RFP with regard to mOSM. MEF cells were stimulated for 20 min at 37 °C with 5 ng/ml mOSM in the presence or absence of mLIF-RFP. Subsequently, cell lysates were analyzed as described above.

the presence or absence of the respective LIF inhibitor. Human and murine LIF are equally potent with regard to the strength of STAT3 tyrosine phosphorylation in MEF cells. Whereas the addition of the inhibitors in a molar ratio of 1:1 to LIF already leads to a decrease of STAT3 tyrosine phosphorylation, addition of the inhibitors in a molar ratio of 5:1 over LIF reduces STAT3 phosphorylation to nearly basal levels. Furthermore, neither mLIFR(D1–D5) nor mLIF-RFP alters the STAT3 phosphorylation after stimulation with hLIF and shL-6Rα, which also signal through gp130. This finding supports the specificity of both inhibitors.

**mLIFR(D1–D5) Specifically Inhibits Human LIF but Does Not Cross-react with Murine LIF**—The inhibitor mLIFR(D1–D5), which includes the domains D1–D5 of the murine LIFR, suppresses the bioactivity of hLIF effectively (Fig. 5A and Fig. 6A). The naturally occurring murine LIF inhibitor LBP (16), which strongly resembles mLIFR(D1–D5), also binds murine LIF but with a considerably lower affinity than human LIF. The binding affinity between mLIFR(D1–D5) and mLIF was not sufficient to show a coimmunoprecipitation at the concentrations we used in the experiment (Fig. 3A). Nevertheless, we wanted to clarify whether mLIFR(D1–D5) is able to suppress the mLIF-induced STAT3 tyrosine phosphorylation in MEF cells. Therefore, MEF cells were stimulated with mLIF in the presence or absence of mLIFR(D1–D5), and the STAT3 tyrosine phosphorylation was analyzed. A 5-fold molar surplus of mLIFR(D1–D5) over mLIF completely inhibits the STAT3 tyrosine phosphorylation in MEF cells (Fig. 5, A and C). By contrast, the addition of mLIFR(D1–D5) in a molar ratio of 1:1 or 5:1 to mLIF did not influence the STAT3 phosphorylation at all (Fig. 5C). This finding supports the results of the coimmunoprecipitations.

**mLIF-RFP Does Not Suppress the STAT3 Tyrosine Phosphorylation upon Stimulation with mOSM—Murine OSM, another site II/III cytokine closely related to LIF, signals through a receptor heterodimer that is composed of OSMR and gp130 (26). It is controversially discussed whether OSM is able to bind to gp130 in the absence of OSMR. The stimulation of an erythroleukemia cell line with human OSM and the simultaneous addition of soluble hgp130 leads to the inhibition of the biological activity of hOSM suggesting a direct interaction between hgp130 and hOSM (27). In contrast, Diveu et al. (28) show that the simultaneous addition of hOSM and shgp130 to Ba/F3-hgp130-hOSMR cells does not inhibit the biological activity of hOSM, and consequently, hOSM does not bind to shgp130. If murine OSM binds to mgp130 in the absence of mOSMR, mLIF-RFP, which contains the CBM of mgp130, could probably inhibit the bioactivity of mOSM. To answer this question, MEF cells were stimulated with mOSM in the presence or absence of mLIF-RFP, and the STAT3 tyrosine phosphorylation was analyzed. As shown in Fig. 5D, a 5-fold molar surplus of mLIF-RFP over mOSM does not inhibit the STAT3 phosphorylation in MEF cells. Accordingly, mOSM does not bind to the CBM of mgp130 in the concentrations we used in the assay. Taken together, mLIF-RFP is a specific inhibitor for mLIF and does not suppress the bioactivity of the related cytokines hIL-6 (Fig. 5B) and mOSM (Fig. 5D).

**mLIFR(D1–D5) and mLIF-RFP Suppress Gene Induction upon Stimulation with hLIF or mLIF, Respectively**—Our next objective was to analyze the effect of mLIFR(D1–D5) and mLIF-RFP on hLIF- or mLIF-mediated gene induction. First, we investigated the inhibition of hLIF-mediated reporter gene induction by mLIFR(D1–D5) in HepG2 cells. In Fig. 6A, HepG2 cells were cotransfected with a reporter gene plasmid coding for luciferase under the control of the STAT3-responsive rat α2-macroglobulin promoter and a β-galactosidase construct with a constitutively active promoter. Afterward, they were stimulated for 16 h with hLIF and an appropriate volume of control supernatant (control-sn) or a combination of hLIF and mLIFR(D1–D5) in the molar ratios as indicated. Subsequently, luciferase activity was measured. Stimulation of the transfected HepG2 cells with 5 ng/ml hLIF leads to a strong increase in luciferase activity. The luciferase activity is blocked to almost basal levels, when mLIFR(D1–D5) is added simulta-
GAPDH mRNA levels served as controls. Taken together, when mLIF-RFP is added at a molar ratio of 1:1 or 5:1. The inhibition of transcription of SOCS3 mRNA to nearly basal levels, whereas mLIF-RFP alone has no effect on SOCS3 mRNA levels. Additionally, mLIF-RFP does not suppress the bioactivity of the closely related cytokine OSM and both inhibitors do not influence the bioactivity of IL-6.

Human LIF was shown to bind to the mLIFR with a surprisingly high affinity (10–20 pM) (17). This high affinity binding of hLIF to mLIFR was described to be the consequence of a lower kinetic dissociation rate compared with that of mLIF. In contrast, hLIF binds to the hLIFR or mLIF to the mLIFR with lower affinity (about 600–2000 pM) (16, 17). The high affinity between hLIF and mLIFR cannot further be increased by the fusion of hgp130(D2–D3) to mLIFR(D1–D5), and consequently hgp130(D2–D3) is not necessary for the inhibition of hLIF. Any truncation of the mLIFR(D1–D5) leads to a loss of function in binding to hLIF (Fig. 2, A and B).

Next, we aimed at clarifying whether it is possible to shorten the mLIFR part in the murine LIF inhibitor being composed of D1 to D5 of mLIFR and the CBM of murine gp130. Because hLIFR and mLIFR share 76% sequence homology (29), and the much higher affinity of mLIFR for hLIF compared with hLIFR can be attributed to distinct amino acid differences all located in the Ig-like domain (30), we wanted to find out whether the results for the human LIF inhibitor are transferable to the murine system. The results of our communoprecipitation experiments between the potential murine LIF inhibitors and murine LIF show that for efficient expression and secretion of the fusion protein or binding of mLIF the domains D1–D5 of mLIFR are all needed (Fig. 3E).

Consequently we support the findings of Owczarek et al. (17) and Voisin et al. (18) who state that the presence of domains D1 to D5 of LIFR is a minimal requirement for proper LIF binding. During the preparation of this manuscript, the crystal structure of hLIF in complex with mLIFR was published (31). According to Huyton et al. (31), mLIFR contacts the site III of human LIF through the Ig-like domain (D3) and loops of the N-terminal half of the C-terminal CBM (D4). Consequently, because D4 contributes to the binding interface between hLIF and mLIFR, its deletion will probably abolish LIF binding. This could be the explanation for the lack of binding of our constructs mLIFR(D1–D3)-hgp130(D2–D3), mLIFR(D3)-hgp130(D2–D3), mLIFR(D1–D3)-mgp130(D2–D3), and mLIFR(D3)-mgp130(D2–D3) to human or murine LIF. Additionally, the complete N-terminal CBM together with the Ig-like domain of hLIFR were shown to be needed for binding of hLIF (18). According to the crystal structure the D2 of mLIFR is of particular importance, although not directly involved in LIF binding. There is an interdomain disulfide bond between the domains.
Novel Inhibitors for Murine and Human LIF

D2 and D3 that fixes the relative orientations of the domains D2 and D3 (31). The deletion of D2 could thus result in a disturbed conformation of the domain D3, explaining the incompetence of the constructs mLIFR(D3–D5)-hgp130(D2–D3) and mLIFR(D3–D5)-mgp130(D2–D3) in binding to LIF. The lack of the domains D1 and D5 of mLIFR in our constructs mLIFR(D2–D4)-hgp130(D2–D3) and mLIFR(D2–D4)-mgp130(D2–D3) also abolishes binding to LIF. As both domains are not directly involved in LIF binding (31), they probably have a function in the correct folding of mLIFR. Therefore, a lack of D1 and D5 could lead to a D2–D4 fragment with a disturbed conformation.

It has already been demonstrated that the natural inhibitor of murine LIF, namely the LBP, a truncated form of the membrane-bound mLIF, binds mLIF with a relatively low affinity (about 600–2000 pm) (16, 17) equally to the membrane-bound mLIF. Fusion of mLIFR(D1–D5) to mgp130(D2–D3) converts LBP into a high affinity binding protein for mLIF (Fig. 3, C and D), which is well suited for the inhibition of the bioactivity of mLIF. This finding is underlined by our comparison between mLIFR(D1–D5) and mLIF-RFP concerning their abilities to inhibit the STAT3 phosphorylation in MEF cells after stimulation with mLIF. In this case, only mLIF-RFP was capable to inhibit STAT3 tyrosine phosphorylation.

IL-6-type cytokines can be subdivided in those containing three (I, II, and III) or two (II and III) receptor-binding sites. Whereas the IL-6 inhibitor IL-6-RFP (2, 3) was designed in our group to target a cytokine containing all three receptor-binding sites, there are many cytokines signaling through a receptor heterodimer, which only binds to the site II and III of the cytokine. LIF, IL-27, IL-12, IL-23, OSM, leptin, and IL-31 belong to this latter group (32). Our main objective behind the construction of human and murine LIF inhibitors based on the ligand-binding domains of their soluble receptors was the construction of prototypical site II/III inhibitors blocking cytokines that do not seem to have a functional site I. Are both our inhibitors mLIFR(D1–D5) and mLIF-RFP really prototypes for the construction of other site II/III inhibitors? For mLIFR(D1–D5), this is rather not the case, because the high affinity binding of hLIF to mLIF is a special situation which probably does not often occur in nature. We propose that mLIF-RFP is really an appropriate prototype for the construction of other site II/III inhibitors that will block the cytokines mentioned above.

Apart from being a prototypical site II/III inhibitor, an inhibitor for LIF could possibly be used as a therapeutic tool for the treatment of rheumatoid arthritis. It was shown that not only the cytokines TNFα, IL-1, IL-6, and interferon-γ (33) but also LIF and OSM are found at elevated concentrations in synovial fluids of patients with rheumatoid arthritis (34–36). Because the treatment of rheumatoid arthritis with a dimeric form of the soluble TNF receptor 1 (or with a humanized IL-6 receptor antibody was not successful in more than 20% of the patients (37), other cytokines such as LIF or OSM might play a pivotal role in maintaining disease progression (38). Possibly, a LIF inhibitor could be utilized for the treatment of rheumatoid arthritis in cases where other therapeutics had no or only a weak effect. This is supported by one study in which the two LIF antagonists LIF05 and MH35-BD, both mutant forms of LIF, were shown to have therapeutic potential for the treatment of rheumatoid arthritis in vitro (38).

Furthermore, a LIF antagonist could become a new nonsteroidal approach for contraception. Blastocyst implantation in mice depends on maternal expression of leukemia inhibitory factor (39). Additionally, the endometrium of infertile women secreted much less LIF than the endometrium of fertile women suggesting a causal relationship between LIF deficiency and implantation failure (40–42). This was further underlined by the finding that maximal LIF concentrations in the human endometrium are present during implantation (43). It is suggested that LIF mediates the interaction between maternal decidual leukocytes and the invading trophoblast (44).

The construction of site II/III inhibitors presented in this study is generally applicable for the development of other cytokine inhibitors, which are based on the fusion of the receptor domains important for cytokine binding. These site II/III inhibitors will be useful for the investigation of the functions of the cytokines or as therapeutic tools for the treatment of diseases caused by dysregulated cytokine expression.

REFERENCES

1. Goldenberg, M. M. (1999) Clin. Ther. 21, 75–87
2. Ancey, C., Küster, A., Haan, S., Herrmann, A., Heinrich, P. C., and Müller-Newen, G. (2003) J. Biol. Chem. 278, 16968–16972
3. Metz, S., Wiesinger, M., Vogt, M., Lauks, H., Schmalzing, G., Heinrich, P. C., and Müller-Newen, G. (2003) J. Biol. Chem. 282, 1238–1248
4. Economides, A. N., Carpenter, L. R., Rudge, J. S., Wong, V., Koehler-Stec, E. M., Hartnett, C., Pyles, E. A., Xu, X., Daly, T. J., Young, M. R., Fandl, J. P., Lee, F., Carver, S., McNay, J., Bailey, K., Ramakanth, S., Hutabarat, R., Huang, T. T., Radziejewski, C., Yancopoulos, G. D., and Stahl, N. (2003) Nat. Med. 9, 47–52
5. Heinrich, P. C., Behrmann, I., Müller-Newen, G., Schaper, F., and Graeve, L. (1998) Biochem. J. 334, 297–314
6. Grötzinger, J., Kurapkat, G., Wollmer, A., Kalai, M., and Rose-John, S. (1997) Proteins 27, 96–109
7. Simpson, R. J., Hammersch, A., Smith, D. K., Matthews, J. M., and Ward, L. D. (1997) Protein Sci. 6, 929–955
8. van Dam, M., Müllerberg, I., Schooltink, H., Stoyan, T., Brakenhoff, J. P., Graeve, L., Heinrich, P. C., and Rose-John, S. (1993) J. Biol. Chem. 268, 15285–15290
9. Giese, B., Au-Yeung, C. K., Herrmann, A., Diefenbach, S., Haan, C., Kuster, A., Wortmann, S. B., Roderburg, C., Heinrich, P. C., Behrmann, I., and Müller-Newen, G. (2003) J. Biol. Chem. 278, 39205–39213
10. Hermanns, H. M., Radtke, S., Haan, C., Schmitt-Van de Leur, H., Tavernier, J., Heinrich, P. C., and Behrmann, I. (1999) J. Immunol. 163, 6651–6658
11. Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Müller-Newen, G., and Schaper, F. (2003) Biochem. J. 374, 1–20
12. Boulanger, M. J., Bankowich, A., J. Kortemme, T., Baker, D., and Garcia, K. C. (2003) Mol. Cell 12, 577–589
13. Gearing, D. P., Thut, C. J., VandeBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D., and Beckmann, M. P. (1993) EMBO J. 12, 2839–2848
14. Fukunaga, R., Ishizaka-Ikeda, E., Pan, C. X., Seto, Y., and Nagata, S. (1992) EMBO J. 19, 877–896
15. Horsten, U., Hermanns, H. M., Radtke, S., Haan, C., Schmitt-Van de Leur, H., Tavernier, J., Heinrich, P. C., and Behrmann, I. (1999) J. Immunol. 163, 6651–6658
16. Hermanns, H. M., Radtke, S., Haan, C., Schmitt-Van de Leur, H., Tavernier, J., Heinrich, P. C., and Behrmann, I. (1999) J. Immunol. 163, 6651–6658
17. Hermanns, H. M., Behrmann, I., Haan, S., Hermanns, H. M., Müller-Newen, G., and Schaper, F. (2003) Biochem. J. 374, 1–20
18. Boulanger, M. J., Bankowich, A., J. Kortemme, T., Baker, D., and Garcia, K. C. (2003) Mol. Cell 12, 577–589
19. Gearing, D. P., Thut, C. J., VandeBos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D., and Beckmann, M. P. (1993) EMBO J. 12, 2839–2848
20. Fukunaga, R., Ishizaka-Ikeda, E., Pan, C. X., Seto, Y., and Nagata, S. (1992) EMBO J. 11, 877–896
21. Horsten, U., Hermanns, H. M., Radtke, S., Haan, C., Schmitt-Van de Leur, H., Müller-Newen, G., and Rose-John, S. (1995) FEBS Lett. 360, 43–46
22. Layton, M. J., Cross, B. A., Metcalf, D., Ward, L. D., Simpson, R. J., and Nicola, N. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8616–8620
23. Owczarek, C. M., Zhang, Y., Layton, M. J., Metcalf, D., Roberts, B., and Nicola, N. A. (1997) J. Biol. Chem. 272, 23976–23985
24. Voisin, M. B., Bitard, J., Daburon, S., Moreau, J. F., and Taupin, J. L. (2002) J. Biol. Chem. 277, 13682–13692
19. Aasland, D., Oppmann, B., Grötzinger, J., Rose-John, S., and Kallen, K. J. (2002) J. Mol. Biol. 315, 637–646
20. He, W., Gong, K., Zhu, G., Smith, D. K., and Ip, N. Y. (2002) FEBS Lett. 514, 214–218
21. He, W., Gong, K., Smith, D. K., and Ip, N. Y. (2005) FEBS Lett. 579, 4317–4323
22. Arcone, R., Pucci, P., Zappacosta, F., Fontaine, V., Malorni, A., Marino, G., and Ciliberto, G. (1991) Eur. J. Biochem. 198, 541–547
23. Aarden, L. A., De Groot, E. R., Schaap, O. L., and Lansdorp, P. M. (1987) Eur. J. Immunol. 17, 1411–1416
24. Weiergrabé, O., Hemmann, U., Küster, A., Müller-Newen, G., Schneider, J., Rose-John, S., Kurschat, P., Brakenhoff, J. P., Hart, M. H., Stabel, S., and Heinrich, P. C. (1995) Eur. J. Biochem. (Tokyo) 234, 661–669
25. Ogata, H., Kobayashi, T., Chinen, T., Takaki, H., Sanada, T., Minoda, Y., Koga, K., Takaesu, G., Maehara, Y., Iida, M., and Yoshimura, A. (2006) Gastroenterology 131, 179–193
26. Ichihara, M., Hara, T., Kim, H., Murate, T., and Miyajima, A. (1997) Blood 90, 165–173
27. Modrell, B., Liu, J., Miller, H., and Shoyab, M. (1994) Growth Factors 11, 81–91
28. Diveu, C., Venereau, E., Froger, J., Ravon, E., Grimaud, L., Rousseau, F., Chevalier, S., and Gascan, H. (2006) J. Biol. Chem. 281, 36673–36682
29. Layton, M. J., Lock, P., Metcalf, D., and Nicola, N. A. (1994) J. Biol. Chem. 269, 17048–17055
30. Bitard, J., Daburon, S., Duplomb, L., Blanchard, F., Vuisio, P., Jacques, Y., Godard, A., Heath, J. K., Moreau, J. F., and Taupin, J. L. (2003) J. Biol. Chem. 278, 16253–16261
31. Huynon, T., Zhang, J. G., Luo, C. S., Lou, M. Z., Hilton, D. J., Nicola, N. A., and Garrett, T. P. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 12737–12742
32. Boulay, J. L., O’Shea, J. J., and Paul, W. E. (2003) Immunity 19, 159–163
33. Feldmann, M., Brennan, F. M., and Maini, R. N. (1996) Annu. Rev. Immunol. 14, 397–440
34. Lotz, M., Moats, T., and Villiger, P. M. (1992) J. Clin. Invest. 90, 888–896
35. Schlaak, J. F., Pfers, I., Meyer Zum Büschenfelde, K. H., and Marker-Hermann, E. (1996) Clin. Exp. Rheumatol. 14, 155–162
36. Waring, P. M., Carroll, G. J., Kandiah, D. A., Buirski, G., and Metcalf, D. (1993) Arthritis Rheum. 36, 911–915
37. Nishimoto, N., Yoshizaki, K., Miyasaka, N., Yamamoto, K., Kawai, S., Takeuchi, T., Hashimoto, J., Azuma, J., and Kishimoto, T. (2004) Arthritis Rheum. 50, 1761–1769
38. Jazayeri, J. A., De Weerd, N., Raye, W., Kivivuori, S., Zabihi, E., and Carroll, G. J. (2007) J. Interferon Cytokine Res. 27, 281–289
39. Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S. J. (1992) Nature 359, 76–79
40. Delage, G., Moreau, J. F., Taupin, J. L., Freitas, S., Hambartsoumian, E., Olivennes, F., Fanchin, R., Letur-Konirsch, H., Frydman, R., and Chaouat, G. (1995) Hum. Reprod. (Oxf.) 10, 2483–2488
41. Hambartsoumian, E. (1998) Am. J. Reprod. Immunol. 39, 137–143
42. Piccinni, M. P., Beloni, L., Livi, C., Maggi, E., Scarselli, G., and Romagnani, S. (1998) Nat. Med. 4, 1020–1024
43. Laird, S. M., Tuckerman, E. M., Dalton, C. F., Dunphy, B. C., Li, T. C., and Zhang, X. (1997) Hum. Reprod. (Oxf.) 12, 569–574
44. Sharkey, A. M., King, A., Clark, D. E., Burrows, T. D., Jokhi, P. P., Charnock-Jones, D. S., Loke, Y. W., and Smith, S. K. (1999) Biol. Reprod. 60, 355–364
45. Varghese, J. N., Moritz, R. L., Lou, M. Z., Van Donkelaar, A., Ji, H., Ivancic, N., Branson, K. M., Hall, N. E., and Simpson, R. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15959–15964