Forced Dimerization of gp130 Leads to Constitutive STAT3 Activation, Cytokine-independent Growth, and Blockade of Differentiation of Embryonic Stem Cells

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The mode of activation of glycoprotein 130 kDa (gp130) and the transmission of the activation status through the plasma membrane are incompletely understood. In particular, the molecular function of the three juxtamembrane fibronectin III-like domains of gp130 in signal transmission remains unclear. To ask whether forced dimerization of gp130 is sufficient for receptor activation, we replaced the entire extracellular portion of gp130 with the c-jun leucine zipper region in the chimeric receptor protein L-gp130. On expression in cells, L-gp130 stimulates ligand-independent signal transducer and activator of transcription (STAT) 3 and extracellular signal-regulated kinase 1/2 phosphorylation. gp130 activation could be abrogated by the addition of a competing peptide comprising the leucine zipper region of c-fos. When stably expressed in the interleukin-3–dependent Ba/F3 murine pre-B-cells, these cells showed constitutive STAT3 activation and cytokine-independent growth over several months. Because gp130 stimulation completely suppressed differentiation of murine embryonic stem cells *in vitro*, we also stably expressed L-gp130 in these cells, which completely blocked their differentiation in the absence of cytokine stimulation and was consistent with high constitutive expression levels of the stem cell factor OCT-4. Thus, L-gp130 can be used *in vitro* and *in vivo* to mimic constitutive and ligand-independent activation of gp130 and STAT3, the latter of which is frequently observed in neoplastic diseases.

INTRODUCTION

The interleukin (IL)-6 family of cytokines consists of IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), Oncostatin M (OSM), IL-27, and new neurotrophin-1 (NNT-1). IL-6 and IL-11 induce the formation of a glycoprotein 130 kDa (gp130)-homodimer, whereas signaling by LIF, CNTF, CT-1, and NNT-1 results in the formation of a gp130/leukemia inhibitory factor receptor (LIFR) heterodimer. OSM can induce the formation of a dimer of gp130 with LIFR and the related protein OSMR (Taga and Kishimoto, 1997). IL-27 exclusively signals via a heterodimer composed of gp130 and the WSX-1 receptor (Pflanz *et al.*, 2004).

IL-6 has pro- and anti-inflammatory properties and plays a central role in host defense against infection and tissue

injuries (Simpson *et al.*, 1997). The activities of IL-6 are highly pleiotropic, stimulating a wide range of biological activities, including B-cell maturation, hepatocyte regeneration, and neuronal growth (Kishimoto *et al.*, 1995).

IL-6 binds to the specific IL-6 receptor (IL-6R), and this complex associates with two molecules of the ubiquitously expressed gp130 (Taga and Kishimoto, 1995; Heinrich *et al.*, 2003). The formation of a hetero-oligomeric receptor complex containing the gp130 signal transducing receptor leads to intracellular activation of Janus tyrosine kinase (JAK)/Tyk tyrosine kinases as well as the signal transducer and activator of transcription (STAT) family of transcription factors. Furthermore, gp130 activation leads to stimulation of the phosphoinositide 3-kinase and RAS/mitogen-activated protein kinase (MAPK) pathways (Hibi *et al.*, 1996). Interestingly, cells that do not express a membrane bound IL-6R and that are therefore not responsive to IL-6, can be stimulated by IL-6 binding to a soluble form of the IL-6R, which is produced by limited proteolysis or translation from a differentially spliced mRNA. This process has been named trans-signaling (Rose-John and Heinrich, 1994).

The extracellular part of gp130 consists of an immunoglobulin (Ig)-like domain followed by a cytokine binding domain and three fibronectin III domains. The binding of the IL-6/IL-6R complex to the Ig-like and cytokine binding domain of gp130 is well understood (Boulanger *et al.*, 2003). The gp130 dimer is formed by contact of gp130 to the binding sites II and III (Ehlers *et al.*, 1994), whereas the IL-6R contacts IL-6 via the binding sites I (Boulanger *et al.*, 2003).

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Abbreviations used: CNTF, ciliary neurotrophic factor; ES, embryonic stem; GFP, green fluorescent protein; Gp130, glycoprotein 130 kDa; IL, interleukin; JAK, Janus tyrosine kinase; LIF, leukemia inhibitory factor; NNT-1, new neurotrophin-1; OSM, oncostatin M; R, receptor; S, soluble; STAT, signal transducer and activator of transcription.

The role of the three fibronectin III domains is less clear. Truncation of these domains results in gp130 molecules devoid of signaling capacity (Kurth *et al.*, 2000). Because all cytokine receptors, which are activated by ligand binding via sites II and III, contain fibronectin III domains, it has been speculated that these domains are needed to position the transmembrane domains of gp130 in proximity (Skinotits *et al.*, 2005).

The dimerization domain of Jun transcription factor proteins are α -helical structures characterized by a periodic repeat of leucine every seven amino acids, i.e., every two helical turns, which forms a parallel coiled coil structure (O'Shea *et al.*, 1989; Glover and Harrison, 1995). Such leucine zippers are found in transcription factors, including c-fos and c-jun, which form the adaptor protein-1 transcription complex. Naturally, Fos and Jun zippers form heterodimers, but they also can form stable homodimers albeit with significantly lower stability. The Jun-Fos leucine zipper has previously been used to dimerize scFv antibodies (de Kruijff and Logtenberg, 1996), interferon γ receptor β subunit (Patel *et al.*, 1996) and the receptors for the cytokines granulocyte-macrophage colony stimulating factor and growth hormone, which both use receptor binding sites I and II (Patel *et al.*, 1996; Behncken *et al.*, 2000).

MATERIALS AND METHODS

Cells and Reagents

COS-7, Ba/F3 (Palacios and Steinmetz, 1985), and HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). Ba/F3-gp130 cells were from Immunex (Seattle, WA) (Gearing *et al.*, 1994), and Phoenix-Eco cells were from our cooperative Prof. Klingmüller (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively. All cells were grown in DMEM high glucose culture medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS), 60 mg/l penicillin, and 100 mg/l streptomycin at 37°C with 5% CO₂ in a water-saturated atmosphere. For the cultivation of Ba/F3 cells, the standard DMEM medium was supplemented with 10% FCS, 60 mg/l penicillin, 100 mg/l streptomycin, and 10% conditioned medium from WEHI-3B cells as a source of IL-3. Ba/F3-gp130 cells were cultured in the presence of 10 ng/ml Hyper-IL-6. Hyper-IL-6 was expressed and purified as described previously (Fischer *et al.*, 1997). Anti-STAT5- and anti-gp130 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-STAT3 was from Transduction Laboratories (Lexington, KY); anti-FLAG M2 was from Sigma-Aldrich (St. Louis, MO); anti-phosphotyrosine monoclonal antibody (mAb) 4G10 was from Upstate Biotechnology (Lake Placid, NY); anti-phospho-STAT3 was from New England Biolabs (Schwalbach, Germany); donkey- α -mouse-AlexaFluor488 and wheat germ agglutinin-tetramethylrhodamine were from Invitrogen (Karlsruhe, Germany); and anti-phospho-extracellular signal-regulated kinase (ERK)1/2 and anti-ERK were from Cell Signaling Technology (Danvers, MA). All restriction enzymes were obtained from MBI Fermentas (St. Leon-Rot, Germany). Tran³⁵S-label and [³H]thymidine were purchased from Amersham Biosciences (Freiburg, Germany).

Construction and Expression of L-gp130 Expression Plasmids

The standard cloning procedures were performed as described previously (Sambrook *et al.*, 1989). For the gp130 hybrid receptor constructs, we added the FLAG-sequence (DYKDDDDK) downstream of the signal peptide (nucleotides 1–66) of the human wild-type gp130. Further downstream, a linker was introduced (for L-gp130, ELGGG and for Δ cys-L-gp130, ELGGG) followed by a 39-aa fragment of the human *c-jun* gene (RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMN) (O'Shea *et al.*, 1989; Patel *et al.*, 1996). The transmembrane domain and the cytoplasmic domain of the wild-type gp130, corresponding to the nucleotides 2068–3007 was added downstream. For Δ -gp130, the 39-aa fragment of the human *c-jun* gene was deleted. For expression studies, L-gp130 and Δ -gp130-transfected cells were cultured for 48 h and metabolically labeled with 50 μ Ci/ml [³⁵S]methionine/[³⁵S]cysteine (Tran³⁵S-Label) in methionine/cysteine-free medium for 2 h. The cells were lysed, and after immunoprecipitation, the complexes were isolated with beads coupled to protein A, subjected to SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes.

Transfection, Transduction, and Selection of Cells

COS-7 and HepG2 cells were transfected by DEAE-dextran technique as described previously (McMahan *et al.*, 1991). HeLa cells were transfected by

ExGen technique, according to manufacturer's instructions (BD Biosciences Clontech, Palo Alto, CA). Ba/F3-, Ba/F3-gp130 cells were transduced retrovirally. The retroviral expression plasmid pMOWS have been described previously (Ketteler *et al.*, 2002). pMOWS-L-gp130, pMOWS- Δ cys-L-gp130, and the control vector pMOWS-GFP (5 μ g each) were transiently transfected in Phoenix-Eco cells (grown on a 6-well plate) by calcium phosphate precipitation as described previously (Ketteler *et al.*, 2002) and according to manufacturer's instructions (BD Biosciences Clontech). The transfection efficiency was visualized after 24 h by green fluorescent protein (GFP) expression (Axiovert 200 Microscope; Carl Zeiss, Jena, Germany). Transducing supernatants were produced as described previously (Ketteler *et al.*, 2002). Two hundred fifty microliters of the transducing supernatants was applied to 1×10^5 Ba/F3 cells, mixed and centrifuged at 1800 rpm for 2 h at 37°C. Afterward, cells were grown in standard medium supplemented with 10% WEHI. Pools of Ba/F3 cells expressing the L-gp130 or the Δ cys-L-gp130-chimera were selected in 1.5 μ g/ml puromycin (Sigma-Aldrich) 48 h after transduction without any cytokine. Surviving cells were daily observed, resulting in Ba/F3-L-gp130 and Ba/F3- Δ cys-L-gp130 cells growing independently from cytokines.

Surface Biotinylation

COS-7 cells were washed four times with phosphate-buffered saline (PBS). Cells were incubated for 15 min with 5 μ g/ml LC-Sulfo-NHS-(+)-Biotin (Pierce Chemical, Rockford, IL). Biotinylation was stopped with 50 mM NH₄Cl. Cells were washed with PBS, lysed, and frozen. Cell lysates were thawed at 4°C and centrifuged. The supernatants were incubated with BS12 (anti-gp130) (L-gp130, Δ -gp130, and wt-gp130).

Immunoprecipitation

Cells were lysed (lysis buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM Na-F, 3 mM Na₃VO₄, and 1% Brij96 supplemented with the Complete protease inhibitor cocktail tablets [Roche Diagnostics, Mannheim, Germany]), scraped off the dishes, and quick frozen in liquid nitrogen before thawing at 4°C on a shaker. Lysates were centrifuged and cleared supernatants were immunoprecipitated with 1–2 μ g of the indicated antibodies overnight at 4°C followed by addition of 50 μ l of protein A-Sepharose (50% slurry) for at least 1 h at 4°C. Immunoprecipitates were washed two times (with lysis buffer but only with 0.1% Brij96) and one time with Tris 20 mM, pH 8, before addition of sample buffer and boiling at 95°C for 5 min.

Immunoblotting and Enhanced Chemiluminescence (ECL) Detection

Immunoprecipitated proteins separated by SDS-PAGE were transferred to PVDF membranes by a semidry electroblotting procedure. Membranes were blocked in a solution of Tris-buffered saline (10 mM Tris, pH 8, and 150 mM NaCl) supplemented with 0.02% Tween and 5% skimmed milk powder for at least 1 h, probed with indicated antibodies overnight at 4°C, and incubated with horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected by chemiluminescence using the ECL kit (Amersham Biosciences) following the manufacturer's instructions.

DNA-binding Protein Assay

HepG2 cells were left untransfected or were transiently transfected with plasmids L-gp130 or Δ cysL-gp130. The cells were stimulated with 50 ng/ml IL-6 for 10 min at 37°C after a serum starvation for 4 h or left unstimulated for controls. Whole cell extracts were prepared as described above. The streptavidin agarose beads were preincubated for 1 h with 2 μ g of double-strand 5'-biotinylated oligonucleotide. Specificity of the observed signals was controlled by introducing in IL-6-activated cell extracts a 30-fold excess of unlabeled double-strand DNA as competitor for the biotinylated oligonucleotide. Solubilized proteins were then incubated with the streptavidin agarose beads overnight at 4°C. Immunoprecipitated complexes were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Detection by immunoblotting was performed with an anti-STAT3-phosphotyrosine mAb followed by ECL (Amersham Biosciences). STAT3 high-affinity interacting motif SIEM67 was derived from the *c-fos* gene (*cis*-inducible element: SIEM67-sense, CATTCCCGTAAATCTTGTCG; SIEM67-reverse, CGACAAGATTT ACGG-GAAATG) (Sadowski *et al.*, 1993; Beadling *et al.*, 1996; Giraud *et al.*, 2001).

Luciferase Assay

HepG2 cells were transiently transfected with expression vectors encoding L-gp130 and Δ -gp130 along with the SIEM-Luc reporter gene vector (kindly provided by Prof. Gascan (INSERM U564, Centre Hospitalier Universitaire d'Angers, Angers, France) (Auguste *et al.*, 1997) and the *Renilla* luciferase-encoding vector pRL-Tk. Forty-eight hours posttransfection, cells were washed and deprived of serum. After another 48 h, cells were stimulated with 50 ng/ml IL-6 or left unstimulated. After an additional 15–18 h, cell lysis and luciferase activity measurement was performed following the manufacturer's instructions (Dual-Luciferase Reporter Assay system; Promega Madison, WI). Firefly luciferase expression (Siem-Luc) was normalized to constitutive *Renilla* luciferase expression (pRL-Tk), determined in parallel.

Proliferation Assays

Ba/F3-, Ba/F3-gp130-, BaF/3-L-gp130, BaF/3-gp130-L-gp130, and BaF/3-gp130- Δ cys-L-gp130 cells were washed three times and resuspended in cytokine-free medium at 5×10^3 cells/well of a 96-well plate. They were cultured in a final volume of 100 μ l with cytokines as indicated in the figure legend for 72–96 h and subsequently pulse labeled with 0.25 μ Ci of [3 H]thymidine for 4 h. Cells were harvested on glass filters and incorporated [3 H]thymidine was determined by scintillation counting. Bioassays were performed with each value being determined in triplicate.

Flow Cytometry Analysis. For analysis, cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS and 0.2% bovine serum albumin) and then incubated at 3×10^5 cells/100 μ l in 20 μ g/ml anti-FLAG M1 antibody in buffer for 60 min on ice. Anti-FLAG antibody was purchased from Sigma-Aldrich. After a single wash in buffer, cells were incubated at 3×10^5 cells/100 μ l in a 1:100 dilution of allophycocyanin conjugated anti-mouse-antibody (Dianova, Hamburg, Germany). Cells were washed once, resuspended in 500 μ l, and analyzed by flow cytometry (BD FACSCanto; BD Biosciences, Franklin Lakes, NJ).

Fos Peptide

The following peptide sequence was synthesized: Fos, N-LTDTLQAET-DQLEDKKSALQTEIANLLKEKEKLEFILAAAY-C (Patel *et al.*, 1996). Lyophilized peptides were solubilized in PBS to a final concentration of 2 mM, and aliquots were stored at -80°C .

Generation of L-gp130-Expressing Embryonic Stem (ES) Cell Lines. ES cells, derived from the parental line W9.5, were routinely grown in the absence of feeder cells in ES cell medium (DMEM containing 15% FCS and 0.1 mM 2-mercaptoethanol) and supplemented with 1000 U/ml LIF (Chemicon International, Temecula, CA) as described previously (Ernst *et al.*, 1994; Starr *et al.*, 1997). Stable transfectants were obtained following coelectroporation (Bio-Rad Gene Pulser set at 250 V and 500 μ F) of the expression plasmid comprising the L-gp130 cDNA inserted in the pEF-BOS plasmid backbone and the resistance marker plasmid pPGKpuropA (Starr *et al.*, 1997). A single cell suspension of electroporated cells was plated at 5×10^3 cells/cm 2 in gelatin-precoated 100-mm-diameter culture plates (Nalge Nunc, Naperville, IL), and 24 h later a 10-d selection period was started in the presence of 1 μ g/ml puromycin (Sigma-Aldrich). Individual puromycin-resistant colonies were analyzed by Western blot analysis for expression of L-gp130, and positive clones expanded by repeated plating of single cell suspension every 4–5 d after trypsinization.

ES Cell Analysis

The extent to which gp130 signaling prevents ES cell differentiation was determined by morphology as described previously (Ernst *et al.*, 1994) and by analysis of Oct-4 expression. All results were confirmed with two independently and clonally derived ES either expressing L-gp130 or the empty pEF-BOS plasmid. Individual ES cell lines were trypsinized, plated at 1×10^3 cells/cm 2 in gelatin-precoated 100-mm-diameter culture plates (Nalge Nunc) in ES cell medium either in the absence of presence of 1000 U/ml LIF, and cultured for up to 6 d. To document cellular morphology, phase contrast photomicrographs of cultures were taken at the indicated time, where pluripotent ES cells look like adherent berry-like balls of tightly packed cells, whereas differentiated cells adopt a spread-out fibroblast-like morphology. Quantitative real-time (RT)-PCR for Oct-4 was carried out on cDNA prepared by the random priming method, using random hexamer oligonucleotide primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), on total RNA extracted by the TRIzol method (Invitrogen) from ES cells cultured as described in the legend to the corresponding figure. We performed quantitative RT-PCR analysis in triplicate in a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia) using SYBR Green (Fisher Biotech, Perth, Australia) with 40 cycles (94°C for 20 s, 62°C for 30 s, and 72°C for 30 s) after initial denaturation at 95°C for 10 min with eukaryotic 18S rRNA serving as a standardize for cDNA concentrations. Data quantification and acquisition of amplification products were carried out with the Rotor-Gene 3000 software on fluorescence resulting from intercalation of SYBR Green dye. Data are expressed in units relative to 18S rRNA expression level. The following set of primers was used for amplification: murine Oct-4-specific primers (Oct4fwd, 5'-CCGGAAGAGAAAGCGAAGCTA; Oct4rev, 5'-TCTCCAGACTCCACCT-CACA); and 18S rRNA-specific primers (M18Sf, 5'-GTAACCCGTTGAAC-CCCATT; M18sr, 5'-CCATCCAATCGGTAGTAGCG).

RESULTS

Design and Construction of Leucin Zipper-containing gp130-Receptors

IL-6 binding to membrane-associated or soluble IL-6R induces dimerization of gp130 (Figure 1A). We have substi-

Figure 1. Schematic membrane organization and idealized activation of gp130 wild-type and gp130 hybrid receptors. (A) Scheme of the gp130 receptor (wt-gp130) with the extracellular domain comprising Ig-like domain (Ig), cytokine binding domain (CBD), three fibronectin type III-like domains (FNIII), transmembrane domain (TM), and cytoplasmic domain (CD), constitutively associated with JAK. Dimerization is induced after binding of IL-6/sIL-6R. (B) gp130 was truncated 15 amino acids above the transmembrane domain and replaced by the leucine zipper (L) region of the human *c-jun* gene and stabilized by an additional disulfide bridge. A FLAG-tag (Flag) was inserted between signal peptide and leucine zipper. (C) The extracellular domain of gp130 was removed and only a FLAG-tag was added (Δ -gp130).

tuted the entire extracellular portion of gp130 by the 39-amino acid Jun leucine zipper sequence of the transcription factor Jun (O'Shea *et al.*, 1989) as schematically illustrated in Figure 1B. To ease immunochemical detection of the chimeric protein, a FLAG-epitope tag was placed immediately NH $_2$ terminal of the leucine zipper sequence. At the junction between the FLAG and leucine zipper, a short glycine linker was introduced to enhance flexibility of the protein, which may facilitate subsequent dimer formation. The transmembrane and cytoplasmic domains of the gp130-receptor were left intact. The resulting chimeric gp130 protein, named L-gp130, consisted of 343 amino acids (Figure 1B). To control for the function of the leucine zipper, a second fusion protein without the leucine zipper, but containing the signal peptide, FLAG-epitope tag, and transmembrane and cytoplasmic domains of the gp130-receptor (Δ -gp130), was constructed (Figure 1C).

Leucin Zipper-gp130 Dimerization Leads to Constitutive, Cytokine-independent Activation of gp130 Signaling

To test for expression of the recombinant proteins, COS-7 cells were transiently transfected with either expression construct and [35 S]methionine/cysteine metabolically labeled chimeric receptors were analyzed by immunoprecipitation followed by fluorography. Figure 2A shows that the L-gp130

Figure 2. Functionality of gp130 mutants in transiently transfected COS-7. (A) To demonstrate expression of gp130 and mutants thereof, COS-7 cells were transiently transfected with 5 μ g of wt-gp130, L-gp130, and Δ -gp130 cDNAs. Forty-eight hours after transfection, cells were metabolically labeled with [35 S]methionine/cysteine. Cells were lysed, and proteins were precipitated with a gp130-specific antibody. Immune complexes precipitated with protein A-Sepharose were separated by SDS-PAGE and visualized by fluorography. Electrophoretic mobilities of molecular mass marker proteins are indicated on the left. (B) Surface biotinylation of COS-7 cells transfected with Δ -gp130, L-gp130, or wt gp130. (C) To probe for cytokine-independent gp130 phosphorylation, COS-7 cells were mock transfected or transiently transfected with 5 μ g of the L-gp130 or Δ -gp130 expression plasmids and lysed 48 h after transfection. Lysates were incubated with a phospho-tyrosine specific antibody (4G10). Precipitated proteins were separated by SDS-PAGE and blotted onto a PVDF membrane. Proteins were detected with a FLAG-specific antibody and visualized by ECL detection.

and Δ -gp130 constructs were expressed as proteins of the expected size of 46 and 36 kDa, respectively, whereas gp130 migrated at 130 kDa. To examine whether the expressed proteins were expressed at the cell surface, we performed surface biotinylation of transiently transfected COS-7 cells. As shown in Figure 2B, only gp130 and the L-gp130 were detected after precipitation with the mAb sc655, which is directed against the intracellular domain of gp130. This result indicates that the gp130 and L-gp130 protein, but not the Δ -gp130 protein, were exposed to the cell surface (Figure 2B). Accordingly, when we analyzed the transfected cells for phosphorylation of the two leucine zipper proteins, it turned out that only the L-gp130 protein was tyrosine phosphorylated (Figure 2C). Apparently the Δ -gp130 protein is not transported to the cell surface and therefore most likely is not functional.

We next tested whether the expression of the leucine zipper gp130 proteins L-gp130 and Δ -gp130 resulted in activation of the transcription factor STAT3 in transiently transfected HepG2 cells. For this purpose, cells were lysed 48 h after transfection, and the lysates were precipitated with the mAb sc655 followed by Western blot analysis with a phosphotyrosine-specific mAb. As can be seen in Figure 3A, expression of the Δ -gp130 did not lead to measurable STAT3 phosphorylation. In contrast, cells expressing L-gp130 showed similar STAT3 phosphorylation as mock-transfected HepG2 cells stimulated with recombinant IL-6 (Figure 3A). Furthermore, L-gp130- and Δ cysL-gp130-transfected cells showed STAT3-specific DNA binding activity (Figure 3B).

To ask whether the leucine zipper gp130 proteins could activate target genes of gp130, HepG2 cells were cotransfected with a reporter construct in which the luciferase gene was placed under the transcriptional control of two STAT3 consensus binding sites upstream of a thymidine kinase minimal promoter (Giraud *et al.*, 2001). As can be seen in Figure 3C, cells transfected with the luciferase construct only showed baseline luciferase activity, which was induced about

Figure 3. STAT3-activation by gp130 mutants in transiently transfected Cos-7 and HepG2-cells. (A) To analyze STAT3 phosphorylation, transiently transfected HepG2 cells expressing the chimeric receptors and mock-transfected cells were serum starved for 48 h after transfection. Cells were stimulated (+) with 50 ng/ml IL-6 for 10 min or left untreated (-). Lysates were prepared and proteins were precipitated with a phospho-specific STAT3 antibody. Equal amounts of proteins were separated by SDS-PAGE and blotted onto a PVDF membrane. Proteins were detected with a STAT3-specific antibody and visualized by ECL detection. (B) To probe for binding of activated STAT3 to cognate DNA recognition sequences, HepG2 cells were cotransfected with the indicated plasmid combinations. Hyper-IL-6 (50 ng/ml) activated or untreated cells were lysed, and extracts were incubated in the presence of a double-strand biotinylated SIEM oligonucleotide. Protein-oligonucleotide complexes were precipitated with streptavidin-agarose beads. The complexes were subjected to SDS-PAGE, and the separated proteins were transferred onto PVDF membranes. Proteins were detected with the anti-phospho-STAT3 antibody and visualized by ECL detection. Specificity of the observed signal was assessed by adding a 30-fold molar excess of a nonbiotinylated SIEM oligonucleotide during incubation. (C) HepG2 cells were transfected with equal amounts of the indicated plasmids along with the reporter gene constructs SIEM-Luc and pRL-Tk. After serum starvation, cells were stimulated with 50 ng/ml IL-6 or were left untreated for 18 h. Cell lysates were prepared as described, and luciferase activity was measured. Luciferase activity of the samples was normalized to the activity of coexpressed *Renilla* luciferase. Representative luciferase activities (RLUs) of five independent experiments (each performed in duplicate) are presented.

fivefold as a consequence of endogenous, wild-type gp130 activation after the administration of recombinant IL-6. By contrast, cotransfection of cells with the L-gp130 leucine zipper gp130 construct resulted in luciferase activity levels comparable with those observed in response to activation of endogenous wild-type gp130 by IL-6 administration (Figure 3C). We conclude from these experiments that the L-gp130 protein, but not the Δ -gp130 protein, was constitutively active, leading to phosphorylation of its cytoplasmic portion as well as of STAT3, and resulting in DNA binding of STAT3 and the subsequent induction of STAT3-dependent target genes.

STAT3 Activation and Cytokine Independent Proliferation of Ba/F3 Cells Stably Transfected with an L-gp130 cDNA

Next, we asked whether installation of L-gp130 into cells would lead to permanent activation of the gp130/STAT3

signaling pathway. The IL-3-dependent murine pro-B-cell line Ba/F3 is the only cell line known not to express gp130. However, these cells are capable to grow in the absence of IL-3 and in the presence of IL-6 when gp130 and IL-6R are supplied by stable transfection (Palacios and Steinmetz, 1985; Gearing *et al.*, 1994; Horsten *et al.*, 1997). We therefore selected Ba/F3 cells as an ideal cellular system to assess the long-term activity of the L-gp130 protein.

We used a retroviral system (Ketteler *et al.*, 2002) to transduce Ba/F3 cells with the cDNA encoding the L-gp130 protein. Cells were transduced and selected with puromycin (see *Materials and Methods*). Resistant cells were placed in medium lacking IL-3. Although untransfected or GFP-transduced cells did not survive in the absence of IL-3, cells transduced with L-gp130 survived and proliferated. We then asked which signaling pathway was activated in control and L-gp130-transduced Ba/F3 cells. In parental Ba/F3 cells treated with either conditioned medium of WEHI-3 cells as a source of murine IL-3, or with recombinant murine IL-3, phosphorylated STAT5 could be detected. No phosphorylated STAT5, the major signaling molecule activated by IL-3, was seen in the absence of IL-3 (Figure 4A). A similar result was obtained in cells stably transfected with a cDNA encoding human wild-type gp130 (Fischer *et al.*, 1997). As expected, treatment of these cells with Hyper-IL-6, a fusion protein of IL-6 and sIL-6R (Fischer *et al.*, 1997), resulted in phosphorylation of STAT3 but not in phosphorylation of STAT5. Similarly, Ba/F3 cells transduced with L-gp130 showed constitutive phosphorylation of STAT3 but not STAT5 in the absence of any cytokine stimulation. These observations are consistent with the well recognized role for STAT3 but not STAT5, in mediating intracellular signaling from gp130. Phosphorylation of the second membrane-proximal tyrosine residue leads to recruitment of a Src homology 2-containing tyrosine phosphatase, SHP2, and subsequent phosphorylation of ERK1/2. Therefore, treatment of Ba/F3-gp130 cells with Hyper-IL-6 induced ERK1/2 phosphorylation, whereas unstimulated cells exhibit no ERK1/2 phosphorylation. As expected, Ba/F3-L-gp130 cells display ERK1/2 phosphorylation without cytokine stimulation (Figure 4B).

Ba/F3 cells transduced with gp130 (Ba/F3-gp130) grow in the presence of Hyper-IL-6 (Fischer *et al.*, 1997). As shown in Figure 4C, in the presence of increasing concentrations of Hyper-IL-6, these cells showed a dose-dependent increase in [³H]thymidine incorporation. Ba/F3 cells transduced with L-gp130 (Ba/F3-L-gp130) showed comparable proliferation to Ba/F3-gp130 cells stimulated with high doses of Hyper-IL-6. Furthermore, Ba/F3-L-gp130 cells continued to grow in the absence of cytokine for more than 8 mo. We conclude from these experiments that the presence of the L-gp130 protein on the cell surface of a heterologous cell system leads to long-term, constitutive activation of gp130 and associated intracellular signaling and target gene activation without cytokine stimulation. Even though Ba/F3-L-gp130 cells exhibit less phosphorylated STAT3 than Hyper-IL-6 stimulated Ba/F3-gp130 cells, the proliferation of both cell lines is comparable. In Ba/F3-gp130-cells, no down-regulation of the STAT-3-phosphorylation status by suppressors of cytokine signaling (SOCS) molecules would be observed, because of the very short treatment of the cells with Hyper-IL-6. Furthermore, it is very likely that the permanent activation of gp130-signal transduction in Ba/F3-L-gp130 cells leads to a SOCS induced down-regulation of phosphorylated STAT3. Conversely, the permanent activation of Ba/F3-gp130 stimulated with Hyper-IL-6 during long-time proliferation might also lead to a slight down-regulation of

Figure 4. Functionality of gp130 mutants in stable transfected Ba/F3 cell lines. (A) Activation of STAT proteins in Ba/F3 cells: to assay for cellular STAT3/STAT5 activation, equal numbers of Ba/F3, Ba/F3-gp130, and Ba/F3-L-gp130 cells after 4-h serum starvation were stimulated for 10 min with 10% conditioned WEHI-medium (IL-3), 10 ng/ml Hyper-IL-6, or were left untreated. (B) Activation of ERK1/2 proteins in Ba/F3 cells: to assay for cellular ERK1/2 activation, equal numbers of Ba/F3-gp130 and Ba/F3-L-gp130 cells after 4 h serum starvation were stimulated for 10 min with 10 ng/ml Hyper-IL-6 or were left untreated. (C) Equal numbers of stably transfected Ba/F3-L-gp130 cells were cultured for 3 d in the presence or the absence of Hyper-IL-6. Proliferation was measured by pulsing the cells after 68 h with [³H]thymidine for 4 h. Cells were harvested and incorporated radioactivity was measured by scintillation counting. Bioassays were performed with each value being determined in triplicate. (D) Equal numbers of stably transfected Ba/F3-L-gp130 cells and nontransfected Ba/F3 cells were lysed with reducing or nonreducing sample buffer. Equal amounts of proteins were separated by SDS-PAGE and blotted onto a PVDF membrane. Proteins were detected with a anti-FLAG-tag-specific antibody and visualized by ECL detection.

phosphorylated STAT-3, resulting in a proliferation comparable with Ba/F3-L-gp130 cells.

Because L-gp130 contains a single cysteine residue in its extracellular portion, we were interested whether the cysteines of two L-gp130 molecules were affiliated with each other. Therefore, we performed an anti-FLAG-tag Western blot analysis of cell lysates from Ba/F3 and Ba/F3-L-gp130 cells under reducing and nonreducing conditions. The molecular mass of L-gp130 was calculated to be 43.4 kDa. As depicted in Figure 4D, monomeric L-gp130 could be clearly detected under reducing conditions and a dimeric form of L-gp130 under nonreducing conditions. We conclude that L-gp130 homodimers are further stabilized by intermolecular disulfide bridges. Two minor bands with a molecular mass of ~40 and 80 kDa most likely represent unspecific binding of the antibody to endogenous cellular proteins because they occur already in the controls.

Leucine Zipper-enforced Dimerization of gp130 Can Be Reversed with a fos Leucine Zipper Peptide

To stabilize the leucine zipper-mediated dimerization of gp130, we had engineered a cysteine residue between the leucine zipper and the FLAG sequence in the L-gp130 protein (Figure 1B). We reasoned that it should be possible to reversibly dissociate the jun leucine zipper used in the L-gp130 protein with the help of a fos leucine zipper peptide (Patel *et al.*, 1996). We therefore first asked whether the presence of the cysteine residue was necessary for the long-term constitutive activation of L-gp130. For these experiments, we used Ba/F3-gp130 cells, because these could be grown as controls in the presence of Hyper-IL-6 to verify that the gp130 signaling pathway was intact after treatment of the cells with Hyper-IL-6 or the fos leucine zipper peptide.

We generated a version of L-gp130, which lacks the cysteine residue (Δ cys-L-gp130). Both, the L-gp130 and the Δ cys-L-gp130 retroviral cDNA constructs were transduced into Ba/F3-gp130 cells. As observed already, puromycin-resistant cells proliferated in the absence of IL-3 (see above).

We determined cell surface expression of the chimeric receptors by flow cytometry (Figure 5A). Cells were stained with anti-FLAG M2 as the primary antibody, followed by a secondary antibody detection step. Both cell lines expressed FLAG-tag containing hybrid receptors on their cell surface at comparable levels. Next, we assessed for STAT3 phosphorylation (Figure 5B). Clearly, in Ba/F3 cells expressing L-gp130 or Δ cys-L-gp130 receptor constructs, constitutively phosphorylated STAT3 protein could be detected at similar levels. Ba/F3-gp130 cells stimulated with Hyper-IL-6 also showed phosphorylated STAT3, whereas in unstimulated Ba/F3-gp130, no phosphorylated STAT3 was detected. Phosphorylated STAT3 protein levels in stimulated Ba/F3-gp130 were about twofold higher than in unstimulated L-gp130- or Δ cys-L-gp130-expressing BAF/3 cells (Figure 5B). The proliferation of the Ba/F3 cells expressing the L-gp130 and the Δ cys-L-gp130 receptor constructs was compared with the proliferation of Ba/F3 cell stably transfected with a human gp130 cDNA. As can be seen in Figure 5C, proliferation of Ba/F3 cells expressing the L-gp130 and the Δ cys-L-gp130 receptor proteins was comparable with the proliferation obtained with the highest Hyper-IL-6 concentration.

Because the Δ cys-L-gp130 protein, in which leucine zipper-mediated dimerization is not stabilized by a disulfide bond, behaved similar to the stabilized L-gp130 protein complex, we asked whether the jun leucine zipper could be destabilized by adding monomeric fos leucine zipper peptide. Thermodynamic analysis had shown that jun-fos heterodimers are more stable than jun homodimers, as a result

Figure 5. Proliferation of BaF/3-gp130- Δ cys-L-gp130 can be inhibited by competitor Fos-peptides. (A) FACS-analysis: the cell lines were labeled as described in *Materials and Methods*. Cell-associated fluorescence was detected by BD FACSCanto (BD Biosciences). Dark gray curves, control cells Ba/F3-gp130; light gray curves, Ba/F3-gp130 cell lines expressing L-gp130 or Δ cys-L-gp130. (B) Activation of STAT proteins in BAF/3 cells: to assay for cellular STAT3 activation, equal numbers of Ba/F3-gp130, Ba/F3-gp130-L-gp130, and Ba/F3-gp130- Δ cys-L-gp130 cells after 4-h serum starvation were stimulated for 10 min with 10 ng/ml Hyper-IL-6 (+) or were left untreated (-). Subsequently, cells were lysed and separated by SDS-PAGE. Proteins were transferred onto PVDF membranes. Proteins were detected with a P-STAT3 and a STAT3-specific antibody and visualized by ECL detection. (C) Equal amounts of stable transfected Ba/F3-gp130-L-gp130, Ba/F3-gp130- Δ cys-L-gp130, and Ba/F3-gp130 cells were cultured for 3 d in the presence or absence of Hyper-IL-6. Proliferation was measured by pulsing the cells after 68 h with [3 H]thymidine for 4 h. Cells were harvested and incorporated radioactivity was measured by scintillation counting. Bioassays were performed with each value being determined in triplicate. (D) Equal amounts of stable transfected Ba/F3-gp130-L-gp130, Ba/F3-gp130- Δ cys-L-gp130, and Ba/F3-gp130 cells were cultured for 3 d in the presence of competitor fos peptide. Proliferation was measured by pulsing the cells after 68 h with [3 H]thymidine for 4 h. Cells were harvested and incorporated radioactivity was measured by scintillation counting. Bioassays were performed with each value being determined in triplicate.

of electrostatic interactions between residues adjacent to the leucines (O'Shea *et al.*, 1989), and the fos peptide has been shown to destabilize a jun homodimer at micromolar peptide concentrations (Patel *et al.*, 1996).

We analyzed the leucine zipper-destabilizing activity of a synthetic 36 amino acid peptide corresponding to the leucine zipper region of the fos protein. The constitutive proliferative activity transduced by the Δ cys-L-gp130 protein expressed in Ba/F3 cells was essentially abolished in the presence of 200 μ M fos-peptide (Figure 5D). Alternatively, the fos peptide-mediated blockage of proliferation could be overcome by treatment of these cells with 10 ng/ml Hyper-IL-6, which induced dimerization of endogenous gp130. This result indicated that the fos peptide at a concentration of 200 μ M had no unspecific cytotoxic effects. Furthermore, proliferation of Ba/F3 cells stably transfected with L-gp130 cells could not be inhibited with 200 μ M Fos-peptide, implying that the leucine zipper in the L-gp130 protein is stabilized by an additional disulfide bridge.

We conclude from these experiments that the jun homodimer in the leucine zipper receptor chimeric protein can be destabilized by the fos leucine zipper peptide and that this destabilization can be used to modulate gp130 signaling pathway activity, which is constitutively activated by the jun leucine zipper in L-gp130.

Use of Leucine Zipper gp130 Receptor Hybrids to Prevent Differentiation of Mouse Embryonic Stem Cells

To test the biological activity of L-gp130 in a relevant homologous cell system, we turned our attention to gp130-mediated regulation of the pluripotent phenotype of mouse ES cells (Evans and Kaufman, 1981; Martin, 1981). Although ES cells undergo spontaneous differentiation when cultured in vitro, their pluripotent character can be maintained by gp130-mediated stimulation as brought about, for example, by administration of LIF, IL-6/sIL-6R, or Hyper-IL-6 (Smith *et al.*, 1988; Nichols *et al.*, 1994; Viswanathan *et al.*, 2002). We therefore asked whether L-gp130 when transfected into ES cells would lead to the maintenance of an undifferentiated state of these cells in the absence of exogenously added gp130 ligands.

Figure 6 shows that ES cells stably transfected with a cDNA coding for the L-gp130 receptor protein maintained a completely undifferentiated phenotype in the absence of the cytokine LIF (Figure 6A, v). By comparison, untransfected wild-type ES cells (Figure 6A, i) cultured for 4 d in the absence of recombinant LIF exhibited a fibroblast-like, differentiated phenotype (Figure 6A, ii), which could be prevented when LIF was included in the culture medium (Figure 6A, iii). As expected, L-gp130-expressing ES cells grown in the presence of LIF also retained a morphologically undifferentiated phenotype (Figure 6A, vi).

Maintenance of an undifferentiated state in embryonic stem cells by the L-gp130 chimeric receptor protein was further confirmed by the quantification of the expression of mRNA coding for the stem cell specific transcription factor Oct-4 (Figure 6B). Here, untransfected cells treated with LIF exhibit high expression of Oct-4, whereas untransfected cells grown in the absence of LIF showed reduced levels of Oct-4 mRNA. By contrast, L-gp130-expressing ES cells, grown either in the absence or presence of LIF, exhibited comparable expression levels of Oct-4 mRNA as wild-type ES cells treated with LIF (Figure 6B).

Collectively, our results in Ba/F3 and murine ES strongly suggest that forced dimerization of gp130 via a leucine zipper is sufficient to activate gp130 to the extent which is

Figure 6. Control of cellular differentiation of embryonic stem cells by L-gp130. (A) Morphological appearance of ES cells expressing L-gp130. Clonally derived ES cell lines expressing L-gp130 (L-gp130) or the empty pEF-BOS expression plasmid (Wt) were routinely maintained in ES cell medium supplemented with 1000 U/ml LIF (+) (i and iv). A single cell suspension of murine Wt ES cells was cultured for 4 d in the presence (iii) or absence (ii) of recombinant LIF, which induces the characteristic change of morphology from densely packed ball-like structure of pluripotent cells to differentiated cells of flattened, fibroblast-like morphology. By contrast, L-gp130-expressing ES cell lines retain their undifferentiated morphology irrespective of the absence (v) or presence (vi) of LIF in the culture medium. (B) Quantitative RT-PCR for Oct-4 expression in murine Wt ES cells or cells expressing L-gp130 (L-gp130) grown for 4 d in the absence (-) or presence of 1000 units/ml LIF (+). RNA extracted from cultures of cells from two independently derived cell lines for each genotype was prepared and analyzed for Oct-4 expression as described previously. Results are expressed as the ratio of Oct-4-18S expression, which served as a control for the amount of RNA analyzed, and data are presented from replicate analysis as the mean $\pm$ SD.

needed to support cytokine-independent growth of pre-B-cells and to maintain an undifferentiated state in ES cells.

DISCUSSION

Here, we describe a novel strategy to enforce constitutive biological activity from gp130, the common receptor subunit for the IL-6 family of cytokines. Our study reveals four major findings. First, replacing the extracellular domain of gp130 by a leucine zipper peptide of the jun protein is

sufficient to dimerize gp130, and thereby leading to phosphorylation of cytoplasmic tyrosine residues of the chimeric receptor protein, subsequent STAT3 and ERK1/2 phosphorylation, and transcriptional activation of gp130 target genes. Second, introduction of the chimeric receptor protein into IL-3-dependent Ba/F3 pro-B-cells confers sustained and long-term factor independence of these cells, suggesting permanent activity of the L-gp130 protein. Third, exploiting the higher stability of a fos-jun heterodimer compared with jun homodimers, we demonstrate that a 36-aa fos leucine zipper peptide can be used to destabilize jun leucine zipper-mediated gp130 dimerization, thereby abolishing the constitutive signaling activity of L-gp130. Last, we show that our novel chimeric receptor protein can be used to maintain the undifferentiated state of murine embryonic stem cells in the complete absence of exogenous cytokine stimulation.

The approach taken assumes that the activation of gp130-associated JAK-kinases is triggered by juxtapositioning of the cytoplasmic tails within gp130 homodimers. Gp130, like other cytokine receptors, has two cytokine binding domains (CBDs) and additional subdomains like an Ig-like and a fibronectin-like (FNIII)-fold. It is believed that during activation, cytokine ligands reorganize their receptor complexes in such a way that the JAKs are correctly juxtaposed, allowing their activation by cross-phosphorylation. There is increasing evidence that many cytokine receptors seem to exist as inactive, preassembled complexes at the cellular surface. This has been demonstrated for the receptors for erythropoietin (Livnah *et al.*, 1999; Remy *et al.*, 1999; Constantinescu *et al.*, 2001); growth hormone (Frank, 2002); interferon- γ (Krause *et al.*, 2002); IL-6 (Schuster *et al.*, 2003); the β_c signaling component in the receptors for IL-3, IL-5, and granulocyte macrophage-colony stimulating factor (Carr *et al.*, 2001); and also for gp130 (Giese *et al.*, 2005; our unpublished data). This implies that ligand binding induces a spatial reorganization within the receptor complex, thereby triggering intracellular signaling.

The contact between IL-6 and the gp130 receptor occurs via the site II and site III on the IL-6 protein. Gp130 contacts these sites via its CBD and the Ig-like domain, which are separated from the plasma membrane by three fibronectin III domains. It is clear that deletion of one or more of the fibronectin III domains leads to receptor molecules that can still bind their ligands but fail to activate intracellular signal transduction (Kurth *et al.*, 2000). This apparently paradoxical situation, although not completely understood, has been illuminated by a recent study by Skiniotis *et al.* (2005) who used single particle electron microscopy to visualize the entire extracellular hexameric IL-6-IL-6R-gp130 complex, containing the Ig-like domain, the CBD, and the three fibronectin III domains. The authors noted that the COOH-terminal portions of the cytokine binding domain are ~100 Å apart. The COOH terminal portions of the membrane proximal fibronectin III domains are dimerized, which would lead to juxtaposition of the transmembrane domains of the cell expressed receptor. In this view, the functional role of the fibronectin III domains would be to assemble the transmembrane domains in proximity. Although we have not addressed this point in our study directly, our results imply that the COOH terminal portion of the membrane proximal transmembrane fibronectin III domains of the activated gp130 dimer need to be positioned in proximity to allow for full activation of the receptor.

In a previous study, we attempted to construct a constitutively active gp130 molecule where dimerization of gp130 was achieved by replacing its entire extracellular portion of the mature gp130 protein with the nine most carboxyl-ter-

minal amino acids from Dind β (Hannemann *et al.*, 1995; Palmer *et al.*, 2005). Although this construct readily conferred STAT3-reporter gene activity, it was unable to sustain factor-independent growth in transfected Ba/F3 cells or to maintain the undifferentiated state of ES cells in the absence of LIF (Ernst, unpublished data).

Our approach to dimerize gp130 with the help of the jun leucine zipper is the first successful strategy to confer constitutive gp130 activation in the complete absence of any extracellular stimulation (Tanner *et al.*, 1995; Li *et al.*, 2002; Kawahara *et al.*, 2004). Moreover, we showed that activation of our gp130 receptor chimeric protein could be abrogated by the addition of a short peptide corresponding to the fos leucine zipper sequence. Our findings add support to the current view that IL-6 induces a conformational change in the extracellular domains of gp130 that is necessary for optimal alignment and binding of the amino acids in the dimerization domain. Finally, this rearrangement stabilizes the dimerized receptor in a conformation that holds gp130-associated JAK kinases in proximity, thereby facilitating signaling.

IL-6/hybridoma growth factor has been shown to be required for growth of murine hybridomas in vivo or in vitro (Mikami *et al.*, 1991). It would be a great advantage in hybridoma technology to develop IL-6-independent hybridoma cell lines by stable transfection of L-gp130 cDNA. The general feasibility of this concept has been shown by Karahara *et al.* (2004) who generated IL-6-independent hybridoma cells stable transfected with a gp130 hybrid receptor, which was inducible by hen egg lysozyme.

Constitutive activation of STAT3, the gp130-dependent transcription factor, has been implicated in many human neoplastic malignancies, including multiple myeloma (Catlett-Falcone *et al.*, 1999; Rawat *et al.*, 2000); prostate cancer, melanoma, ovarian cancer, and renal carcinoma (Bromberg, 2002); and gastric cancer (Jenkins *et al.*, 2005). Artificially dimerized STAT3 was shown to exhibit oncogenic potential, and STAT3 was therefore designated as an oncogene (Bromberg *et al.*, 1999). So far, it is not understood which processes lead to the observed constitutive activation of STAT3. It is not unlikely that in such malignancies, constitutive activation of gp130 either by an autocrine or paracrine loop of stimulating cytokines, e.g., of IL-6 or by genetic alterations, causes the activation of STAT3 (Sriuranpong *et al.*, 2003; Selander *et al.*, 2004). Moreover, a constitutively active gp130 protein provides an excellent molecular tool to establish animal models of diseases in which activated STAT3 is believed to play a role. Therefore, our constitutively active L-gp130 receptor molecule, for example, in conjunction with the reversibly inducible tetracycline expression system (Gossen *et al.*, 1995), may provide an exciting opportunity to generate animal models to assess the consequence of constitutive pathophysiologic activation of STAT3. The potential advantage of using a model based on L-gp130 rather than to rely on a constitutive active STAT3 mutants relates to the observation that the extent of STAT3 activation in the latter case is only a fraction of that observed in tumor cells (Bromberg *et al.*, 1999), whereas the levels of L-gp130-mediated STAT3 activation are comparable with those observed after full stimulation of the wild-type gp130 receptor. The use of L-gp130 is therefore likely to provide novel insight into the consequences of massive and uncontrolled activation of gp130 signaling cascades in vivo, which has been associated with many types of solid and hematological tumors.

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