Constitutively Active Mutant gp130 Receptor Protein from Inflammatory Hepatocellular Adenoma Is Inhibited by an Anti-gp130 Antibody That Specifically Neutralizes Interleukin 11 Signaling*

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Background: Constitutively active, mutant gp130 is responsible for the development of inflammatory hepatocellular adenomas (IHCA).

Results: The anti-gp130 antibody B-P4 blocks constitutive activation of mutant gp130.

Conclusion: B-P4 might be a drug candidate for IHCA and rare cases of gp130-associated hepatocellular carcinoma.

Significance: This is the first report on how to block oncogenic activation of gp130.

Ligand-independent constitutively active gp130 mutants were described to be responsible for the development of inflammatory hepatocellular adenomas (IHCAs). These variants had gain-of-function somatic mutations within the extracellular domain 2 (D2) of the gp130 receptor chain. Cytokine-dependent Ba/F3 cells were transduced with the constitutively active variant of gp130 featuring a deletion in the domain 2 from Tyr-186 to Tyr-190 (gp130ΔYY). These cells showed constitutive phosphorylation of signal transducer and activator of transcription-3 (STAT3) and cytokine-independent proliferation. Deletion of the Ig-like domain 1 (D1) of gp130, but not anti-gp130 mAbs directed against D1, abolished constitutive activation of gp130ΔYY, highlighting that this domain is involved in ligand-independent activation of gp130ΔYY. Moreover, soluble variants of gp130 were not able to inhibit the constitutive activation of gp130ΔYY. However, the inhibition of constitutive activation of gp130ΔYY was achieved by the anti-gp130 mAb B-P4, which specifically inhibits gp130 signaling by IL-11 but not by other IL-6 type cytokines. IL-11 but not IL-6 levels were found previously to be up-regulated in IHCAs, suggesting that mutations in gp130 are leading to IL-11-like signaling. The mAb B-P4 might be a valuable tool to inhibit the constitutive activation of naturally occurring gp130 mutants in IHCAs and rare cases of gp130-associated hepatocellular carcinoma.

Inflammatory hepatocellular adenoma (IHCA) is a subtype of hepatocellular adenoma, which is a rare benign liver tumor mostly affecting younger females. IHCA are characterized by polymorphic inflammatory cell infiltrates and activation of acute phase proteins, such as C-reactive protein and serum amyloid A (1). IHCA show constitutive phosphorylation of signal transducer and activator of transcription 3 (STAT3), indicating a crucial role of gp130 signaling (2). The activation of gp130 receptor complexes leads to intracellular activation of Janus kinases (Jak/Tyk) as well as the STAT family of transcription factors such as STAT1 and STAT3. Furthermore, the activation leads to stimulation of the Ras/Raf/MAP kinase pathways (3). Importantly, the gp130 cytokine family member IL-6 is not overexpressed in IHCA. However, about 60% of the investigated patient samples revealed small in-frame deletions within the binding site II of domain 2 (D2) of gp130 (2) and additional 12% carried activating STAT3 mutations (4). The marked activation of the gp130 signaling pathway in IHCA was shown to be directly caused by these gain-of-function somatic mutations within the gp130 receptor chain, resulting in ligand-independent constitutive active mutant gp130 proteins (2). Sustained ligand-independent activation of gp130 homo- and heterotypic signaling pathways was demonstrated recently, showing that long-term activation was not suppressed by negative feedback loops (5, 6). Mutant gp130 receptor chains were coexpressed along with wild-type gp130, suggesting a dominant effect of the mutations. β-catenin mutations are frequently associated with IHCA (2). The low transformation potential of IHCA (below 5%) might be attributed to the coexistence of gp130-mutations plus activated β-catenin pathways (2).

The presented experiments show that gp130ΔYY also confers ligand-independent and sustained proliferation of Ba/F3-gp130ΔYY cells, which adds gp130 to the list of oncogenes. Moreover, the immunoglobulin-like domain 1 (D1) of gp130 is crucial for the receptor autoactivation of a frequent in-frame gp130 deletion variant spanning a deletion in the domain 2 (D2) from Tyr-186 to Tyr-190 and designated as gp130ΔYY. More-
Blocking Constitutively Active gp130 Signaling

over, we demonstrate the specific and efficient inhibition of autonomous gp130ΔYY receptor activation by the neutralizing anti-gp130 antibody B-P4, which specifically inhibits IL-11-mediated signaling.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Ba/F3 and Ba/F3-gp130 cells were obtained from Immunex (Seattle, WA) (7), COS-7 cells from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and Phoenix-Eco cells from U. Klingmüller (Deutsche Krebsforschungszentrum, Heidelberg, Germany). All cells were grown in DMEM high-glucose culture medium (PAA Laboratories, Colbe, Germany) supplemented with 10% FBS, penicillin (60 mg/liter), and streptomycin (100 mg/liter) at 37 °C with 5% CO2 in a water-saturated atmosphere. For cultivation of Ba/F3-gp130 cells, standard DMEM was supplemented with 10 ng/ml Hyper-IL-6. Hyper-IL-6 is a fusion protein of IL-6 and the soluble IL-6 receptor that mimics IL-6 trans-signaling (8, 9). Hyper-IL-6 was expressed and purified as described previously (8). Ba/F3 cells were cultured in the presence of IL-3 or conditioned medium from WEHI-3B cells, which constitutively produce IL-3. Anti-Myc-tag (71D10), anti-STAT1/3, and anti-phospho STAT1/3 mAbs were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). Anti-c-myc (9E10) mAbs were from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-gp130 mAb B-T2 was obtained from Abcam (Cambridge, UK), anti-gp130 mAb B-R3 from Santa Cruz Biotechnology, and anti-gp130 mAb B-P4 from Hözel (Hözel Diagnostika GmbH, Köln, Germany). The recombinant proteins sgp130 and sgp130Fc were expressed and purified as described previously (10).

Construction of Expression Plasmids—Standard cloning procedures were performed as described (11). The plasmid pBSK-gp130 was used as a template to amplify a fragment of gp130 coding for the deletion from Tyr-186 to Tyr-190 with the primer 5’-GATATCGGCCGCAATGTTGACGCTGACAAGCTTGCTG-3’ and 3’-GACAAATCAACATGCTGAGTTTCTG-3’. The resulting PCR product was subcloned into pBSK-gp130 via HincII (vector) and EcoRV (insert) to obtain the plasmid pBSK-gp130ΔYY.

The deletion of the sequence coding for D1 of gp130 (from proline 27 to glycine 123) was performed by splicing by overlap-extension PCR, which preserved the original signal peptide coding sequence of gp130. The resulting plasmid was named pBSK-gp130ΔD1. For producing the deletion from Tyr-186 to Tyr-190 in the D2 domain of gp130, the plasmid pBSK-gp130ΔD1 was used as a template. Again, the resulting PCR product was subcloned into pBSK-gp130ΔD1 via HincII (plasmid) and EcoRV (insert) to obtain pBSK-gp130ΔD1ΔYY.

The plasmid pEYFP-gp130 (12) was digested with NotI and EcoRI for obtaining the enhanced YFP (EYFP)-tagged C terminus of gp130, which was subcloned into pBSK-gp130ΔYY and pBSK-gp130ΔD1ΔYY. The resulting plasmids were named pBSK-gp130ΔYY-EYFP and pBSK-gp130ΔD1ΔYY-EYFP. These plasmids were used to create plasmids for the expression of C-terminal myc-tagged gp130 expression vectors by exchange of the EYFP with the myc tag (5’ primer 5’-GATCCAGAACAAAACTCATCTCAGAAGAGGTATCTGAGG-3’ and 3’ primer 5’-GGCCCGCTACAGATCCTTCTTCTGAGATGATTTCAGTGTTCTG-3’) using NotI and BamHI. The resulting plasmids were named pBSK-gp130ΔYY-myc and pBSK-gp130ΔD1ΔYY-myc.

The plasmid pBSK-gp130ΔYY was digested with HincII and ligated with an HindIII fragment containing the gp130 signal peptide, myc tag, and a part of the gp130 extracellular domain (synthesized by GENEART, Regensburg, Germany), leading to pBSK-myc-gp130. To obtain the corresponding plasmid containing the deletion from Tyr-186 to Tyr-190, pBSK-myc-gp130 was used as a template to amplify a fragment of gp130 coding for the deletion with the primer 5’-gp130c2 and 3’-gp130delSY. The resulting plasmid was named pBSK-myc-gp130ΔYY. For deletion of D1, pBSK-myc-gp130 was digested with HindII, and a fragment coding for the signal peptide of gp130, a myc-tag, and a part of gp130 containing the deletion of D1 and Tyr-186 to Tyr-190 was inserted, resulting in pBSK-myc-gp130ΔD1ΔYY. cDNAs coding for gp130ΔYY, gp130ΔD1ΔYY, gp130ΔYY-myc, gp130ΔD1ΔYY-myc, myc-gp130, myc-gp130ΔYY, and myc-gp130ΔD1ΔYY were subcloned into the retroviral expression vector pMOWS (13). The resulting plasmids were named pMOWS-gp130ΔYY, pMOWS-gp130ΔD1ΔYY, pMOWS-gp130ΔYY-myc, pMOWS-gp130ΔD1ΔYY-myc, pMOWS-myc-gp130, pMOWS-myc-gp130ΔYY, and pMOWS-myc-gp130ΔD1ΔYY. The vector pMOWS-gp130-myc was obtained by digestion of pMOWS-gp130-EYFP and pMOWS-gp130ΔYY-myc with BpiI and HindIII. Subsequently, the resulting pMOWS vector backbone and the insert containing the C-terminal myc tag were ligated. cDNAs coding for myc-gp130, myc-gp130ΔYY, and myc-gp130ΔD1ΔYY were subcloned additionally into the expression plasmid p409 (14).

Transfection, Transduction, and Selection of Ba/F3-gp130 Cells—The murine pre-B cell line Ba/F3 and Ba/F3-gp130 cells, stably transduced with human gp130, were used for retroviral transduction with the plasmid derivatives of the retroviral expression vector pMOWS. For this purpose, pMOWS plasmids (1 μg each) were transiently transfected in 8 × 105 Phoenix-Eco cells using TurboFect™ according to manufacturer’s instructions (Fermentas, St. Leon-Rot, Germany). The transfection efficiency was typically about 50%, which was estimated by GFP expression 24 h after transfection (Axiovert 200 microscope, Zeiss). Retroviral supernatants were produced as described (13). 250 μl of the retroviral supernatant were applied to 1 × 105 Ba/F3 or Ba/F3-gp130 cells and mixed, and the solution was centrifuged at 1800 rpm for 2 h at 21 °C in the presence of polybrene (8 μg/ml). Transduced cells were grown in standard medium supplemented with either 10 ng/ml IL-3 (Ba/F3 cells) or 10 ng/ml Hyper-IL-6 (Ba/F3-gp130 cells). 48 h after transduction, transduced cells were selected in 1.5 μg/ml puromycin (PAA Laboratories) for at least 2 weeks. After 2 weeks of antibiotic selection in the presence of IL-3 or Hyper-IL-6, the cells were screened for cytokine-independent proliferation.

Proliferation Assays—Transduced Ba/F3-gp130 cells expressing the gp130 variants with or without the myc tag were washed three times with sterile PBS and suspended in DMEM containing 10% FBS at 5 × 105 cells per well of a 96-well plate. The cells were cultured for 3 days in a final volume of 100 μl
with or without additional cytokines or antibodies as indicated. The CellTiter-Blue® cell viability assay (Promega, Mannheim, Germany) was used to determine the cell number following the manufacturer’s instructions and measured on a Lambda Fluoro 320 fluorometer (excitation filter 530/25, emission filter 590/35, sensitivity 75, software KC4). Relative light unit values were normalized by subtractions of negative control values (unstimulated Ba/F3-gp130 cells) from all other values. All values were measured in triplicates.

**Western Blotting**—For detection of phospho-STAT3, cells were washed three times with sterile PBS and starved for 6 h in serum-free DMEM before adding additional cytokines or sgp130Fc as indicated. Subsequently, cells were centrifuged, and the pellet was directly frozen in liquid nitrogen. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 1 mM Na3VO4, 1% IGEPAL (Nonidet P-40) and 1% Triton-X-100, supplemented with complete protease inhibitor mixture tablets (Roche)).

Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBS-T; 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.5% Tween 20) and probed with primary antibodies as indicated at 4 °C overnight. After washing with TBS-T, the membranes were incubated with the appropriate secondary antibodies conjugated to HRP (Thermo Scientific/ Pierce, Perbio), and protein bands were visualized with the ECL detection system (GE Healthcare) according to the manufacturer’s instructions.

**Flow Cytometry Staining and Analysis**—To detect the surface expression of N-terminally myc-tagged gp130 variants, cells were washed with FACS buffer (PBS, 1% BSA) and incubated at 5 × 10^5 cells/100 μl of FACS buffer containing 1:100 diluted anti-myc tag (71D10) mAb (Cell Signaling Technology) in FACS buffer for 60 min on ice. After a single washing step in FACS buffer, cells were incubated in 100 μl of FACS buffer containing a 1:100 dilution of Alexa Fluor 488-conjugated anti-rabbit mAb (Life Technology, Darmstadt, Germany), respectively. Cells were washed once with FACS buffer, resuspended, and analyzed by flow cytometry (BD Biosciences, FACSCantoll and FACS DIVA software). Detection of gp130 on the cell surface was further performed with mouse anti-gp130 (B-R3) mAb (sc-57189, Santa Cruz Biotechnology) followed by allophycocyanin-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgG (Dianova, Hamburg, Germany).

**Coprecipitation Studies Using the Nanotrap System**—For coprecipitation, transiently transfected COS-7 cells were collected by scraping and subsequently lysed in 200 μl of lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 0.5% Nonidet P-40). The volume of the lysate was adjusted to 500 μl with dilution buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF). 50 μl of each lysate was boiled with 50 μl 4X Laemmli buffer. The remaining 450 μl of lysate was incubated with anti-GFP-specific nanobodies (25 μg), coupled to N-hydroxysuccinimide-activated Sepharose as described previously (15). The mixture was incubated at room temperature in an overhead rotator for 2 h. Afterward, the Sepharose was washed four times with 250 μl dilution buffer and subsequently boiled in 100 μl 2x Laemmmli buffer. The lysate and the precipitated proteins were separated by SDS-PAGE and analyzed by Western blotting using mAbs against the myc-tag or GFP and the appropriate secondary antibodies conjugated to horseradish peroxidase (Thermo Scientific). Protein bands were visualized with the ECL detection system (GE Healthcare) according to the manufacturer’s instructions.

**RESULTS**

**Cell-autonomous Proliferation of Ba/F3 Cells by the Ligand-independent, Constitutively Active gp130 Variant gp130ΔYY**—We have generated the ligand-independent constitutively active gp130 variant gp130ΔYY, featuring a deletion in the domain 2 (D2) from Tyr-186 to Tyr-190 (Fig. 1A). This variant was selected because it represents four of 26 identified mutations in IHCAs (2). Moreover, 20 of 26 patients carried deletions that include this region or a deletion from ΔSer-187 to Tyr-190 (gp130ΔSY, six of 26). Therefore, we conclude that amino acids Tyr-186 to Tyr-190 of gp130 are representative for most of the ligand-independent gp130 receptor variants. The murine pre-B cell line Ba/F3 was chosen as a model system to investigate the constitutive activation of gp130ΔYY. Ba/F3 cells usually grow in dependence of the cytokine IL-3. However, transduction with the gp130 receptor chain cDNA, Ba/F3-gp130 cells grow in the presence of IL-6 and the soluble IL-6R or Hyper-IL-6, which is a fusion protein thereof (8, 9). The cDNA encoding gp130ΔYY was stably transduced into Ba/F3-gp130 cells (Ba/F3-gp130-gp130ΔYY) because Ba/F-3 cells expressing the wild-type and the mutated gp130 receptor reflected the in vivo situation, with heterozygous cells having a wild-type and a mutated gp130 allele. Ba/F3-gp130-gp130ΔYY cells showed ligand-independent STAT3 phosphorylation and long-term proliferation, indicating that gp130ΔYY confers a dominant ligand-independent, cell-autonomous gp130 receptor activation phenotype (Fig. 1, B and C). Even though STAT3 phosphorylation has already been shown for transiently transfected Hep3B cells (2), it remained elusive whether gp130ΔYY also mediates long-term receptor activation and cellular proliferation. Because Ba/F3-gp130-gp130ΔYY cells also expressed the wild-type gp130 receptor, it was not possible to prove protein expression of the untagged gp130ΔYY protein in these cells. The size difference between wild-type gp130 and gp130ΔYY was only five amino acids, and both gp130 receptor variants were almost undetectable by Western blotting using anti-gp130 antibodies (data not shown). Therefore, stably transduced Ba/F3-gp130 cells with C-terminally myc-tagged wild-type gp130 and gp130ΔYY proteins (referred to as Ba/F3-gp130-gp130-myc and Ba/F3-gp130-gp130ΔYY-myc) were generated. Expression of the corresponding cDNAs was demonstrated by Western blotting with anti-myc mAbs (Fig. 1D). Again, only gp130ΔYY-myc transduced Ba/F3-gp130 cells showed cytokine-independent proliferation and STAT3 phosphorylation (Fig. 1, E and F).

Interestingly, the wild-type gp130 receptor present in Ba/F3-gp130-gp130ΔYY cells did not interfere with cytokine-independent proliferation and STAT3 activation induced by...
Here, we cannot exclude that the expression of wild-type gp130 receptor was too low to observe inhibition of gp130\textsubscript{YY}. Inhibition of gp130\textsubscript{YY} was demonstrated for Hep3B cells overexpressing both the wild-type and mutant gp130 receptor, albeit with an excess of the wild-type receptor (2). Of note, Ba/F3-gp130-gp130\textsubscript{YY-myc} cells were selected for cytokine-independent growth, thus preferentially selecting clones that express a wild-type gp130/gp130\textsubscript{YY} ratio that promotes gp130\textsubscript{YY} activation.

**Cell-autonomous Proliferation of Ba/F3 Cells by the Ligand-independent, Constitutively Active gp130 Variant gp130\textsubscript{YY}**

The extracellular part of the gp130 receptor consists of one immunoglobulin-like domain (Ig-like, D1), the cytokine binding module (CBM, D2)
and D3), and three fibronectin-like type III domains (FNIII, D4-D6) (16). The binding of IL-6 and IL-11 to gp130 is mediated by the D1 domain of gp130 via site III of IL-6/IL-11 and the CBM of gp130 via site II of IL-6/IL-11 (17). A gp130 receptor lacking the Ig-like domain 1 (gp130ΔD1) is functionally inactive because gp130ΔD1 cannot form homodimers because of the missing binding site III for IL-6/IL-11 (18). To answer the question whether the D1 domain is crucial for the biological activity of gp130ΔYY, we genetically deleted the D1 domain from gp130ΔYY-myc (named gp130ΔD1ΔYY-myc) and generated Ba/F3-gp130-gp130ΔD1ΔYY-myc cells (Fig. 1A). As depicted in Fig. 1D, the gp130ΔD1ΔYY-myc protein was produced by Ba/F3-gp130-gp130ΔD1ΔYY-myc cells. However, Ba/F3-gp130-gp130ΔD1ΔYY-myc cells were not able to proliferate cytokine-independently and showed no constitutive STAT3 phosphorylation (Fig. 1, E and F). Published coexpression studies showed that constitutively active gp130ΔDY interacts with gp130ΔSYY and wild-type gp130 in the absence of a ligand (2). Surprisingly, the biologically inactive variant gp130ΔD1ΔYY was also precipitated with gp130ΔD1ΔYY in the absence of any ligand (Fig. 1G), indicating that gp130ΔD1ΔYY can form inactive homodimers. These results suggested that the D1 domain of gp130ΔYY is crucial for ligand-independent receptor activation.

Using C-terminally tagged variants of gp130, we were not able to show that the gp130ΔD1ΔYY-myc variant was transported to the cell surface. Therefore, N-terminally tagged gp130 variants (myc-gp130, myc-gp130ΔYY, and myc-gp130ΔD1ΔYY) were generated and stably transduced into Ba/F3 and Ba/F3-gp130 cells. We were, however, not able to detect the N-terminal myc-tagged gp130 variants in lysates of Ba/F3 cells by Western blot analysis, but expression was verified in COS-7 cells (Fig. 2A). As expected, only the N-terminally tagged gp130ΔYY conferred ligand-independent proliferation of Ba/F3-gp130-myc-gp130ΔYY cells (Fig. 2B). Moreover, naive Ba/F3 cells stably transduced with myc-gp130, myc-gp130ΔYY, or myc-gp130ΔD1ΔYY were generated and tested for STAT3 phosphorylation with and without Hyper-IL-6 stimulation. Ba/F3-myc-gp130 showed Hyper-IL-6-induced STAT3 phosphorylation, Ba/F3-myc-gp130ΔYY showed STAT3 phosphorylation in the absence of cytokine stimulation, and Ba/F3-myc-gp130ΔD1ΔYY showed no STAT3 phosphorylation irrespective of cytokine stimulation (Fig. 2C). Even though Ba/F3-myc-gp130 cells showed STAT3 phosphorylation after Hyper-IL-6 stimulation, these cells did not shift from IL-3-dependent to Hyper-IL-6-dependent proliferation. However, after long-term cultivation with IL-3, Ba/F3-myc-gp130ΔYY cells converted to ligand-independent proliferation. For unknown reasons, we were not able to adopt the proliferation of Ba/F3-gp130 cells that were freshly transduced with wild-type gp130, to Hyper-IL-6-dependent growth (Fig. 2D).

Finally, cell surface expression of all N-terminally myc-tagged gp130 variants of stably transduced Ba/F3 and Ba/F3-gp130 cells was shown by FACS analysis using an anti-myc mAb, excluding the possibility that the biological inactivity of gp130ΔD1ΔYY was due to inefficient transport to the plasma membrane (Fig. 2E). Furthermore, cell surface expression of gp130 variants of generated Ba/F3 cells was confirmed using the anti-gp130 mAb B-R3 (Fig. 2F).

The Constitutively Active gp130 Variant gp130ΔYY Is Inhibited by the Anti-gp130 Antibody B-P4—The D1 domain was needed for ligand-independent activation of gp130ΔYY. Therefore, we hypothesized that the mAb B-T2, which binds to the D1 domain and inhibits IL-6-induced activation of gp130, might also inhibit gp130ΔYY-induced proliferation (19). As expected, B-T2 inhibited Hyper-IL-6 induced proliferation of Ba/F3-gp130-myc-gp130 cells in a dose-dependent manner, but the proliferation of Ba/F3-gp130-myc-gp130ΔYY was not inhibited (Fig. 3A). From this experiment, we concluded that even though the D1 domain of gp130 was critical for cytokine-independent proliferation mediated by gp130ΔYY, the binding of IL-6 via site III to gp130 was not involved in the constitutive activation of gp130ΔYY.

Because we did not observe an inhibitory effect of wild-type gp130 on gp130ΔYY-induced cell proliferation and STAT3 phosphorylation in Ba/F3-gp130-gp130ΔYY cells, we tested whether the soluble gp130 (sgp130) or the fusion protein sgp130Fc, which both contain all extracellular domains of gp130, inhibit the ligand-independent activation of gp130ΔYY. Sgp130 is thought to be the natural inhibitor of IL-6 trans-signaling via the soluble IL-6-IL-6 receptor complex (10). Hyper-IL-6-induced proliferation of Ba/F3-gp130-gp130-myc cells was inhibited in a dose-dependent manner by sgp130 and sgp130Fc as described previously (Fig. 3, B and C) (10). However, sgp130 and sgp130Fc did not inhibit proliferation of Ba/F3-gp130-gp130ΔYY-myc cells. Furthermore, activation of STAT3 was efficiently inhibited by sgp130Fc in Ba/F3-myc-gp130 cells but not in the corresponding myc-gp130ΔYY cells (Fig. 3D).

Next, we tested two other neutralizing mAbs against gp130 for inhibition of the ligand-independent activation of gp130ΔYY. The mAb B-R3 is directed against the CBM (domain 2 of gp130) (19, 20), whereas the mAb B-P4 binds to the first of three fibronectin domains (domain 4 of gp130) (19, 21). As shown in Fig. 4A, B-R3 inhibited the proliferation of Ba/F3-gp130 cells stimulated with Hyper-IL-6 in a dose-dependent manner. However, the proliferation of Ba/F3-gp130-gp130ΔYY or Ba/F3-gp130-Δgp130 cells was not affected by B-R3. In L-gp130, the entire extracellular portion of gp130 was replaced with the c-jun leucine zipper region (5). As a control, B-R3 did not inhibit the proliferation of Ba/F3-gp130 cells stimulated with IL-3, indicating that B-R3 specifically blocked the receptor activation of gp130 in Ba/F3-gp130 cells (Fig. 4B). The binding epitope of B-R3 is within the CBM (D2). The failure of B-R3 to inhibit gp130ΔYY-induced cellular proliferation cannot be caused by the inability of B-R3 to bind to gp130ΔYY because this mAb was successfully used for detection of gp130, gp130ΔYY, and gp130ΔD1ΔYY in flow cytometry (Fig. 2E).

Interestingly, B-P4 specifically inhibited the proliferation of Ba/F3-gp130-gp130ΔYY in a concentration-dependent manner. Proliferation of Ba/F3-gp130-L-gp130 cells and Hyper-IL-6-induced proliferation of Ba/F3-gp130 cells was not inhibited (Fig. 4C). It was described previously that B-P4 specifically inhibits gp130 receptor activation exclusively induced by IL-11 but not by IL-6 (Hyper-IL-6), leukemia inhibitory factor,
FIGURE 2. Gp130ΔYY and gp130ΔD1ΔYY are presented on the cell surface of stably transduced Ba/F3 cells. A, Western blot detection of myc-tagged gp130ΔYY, gp130ΔD1ΔYY, and gp130 in transiently transfected COS-7 cells using anti-myc-mAbs. GFP-transfected cells served as a negative control. B, equal numbers of Ba/F3-gp130 cells stably transduced with myc-gp130ΔYY, myc-gp130ΔD1ΔYY, myc-gp130, or GFP were cultured for 3 days in the presence or absence of Hyper-IL-6. Proliferation was measured as indicated under “Experimental Procedures.” C, after 6 h of serum starvation, Ba/F3 cells stably transduced with myc-gp130ΔYY, myc-gp130ΔD1ΔYY, or myc-gp130 were stimulated for 5 min with Hyper-IL-6 or left untreated. Cells were analyzed for STAT3 phosphorylation by Western blotting. Membranes were stripped and reprobed with anti-STAT3 mAbs. As a negative control, Ba/F3 cells were used. D, equal numbers of Ba/F3 cells stably transduced with myc-gp130ΔYY, myc-gp130ΔD1ΔYY, or myc-gp130 were cultured for 3 days in the presence or absence of IL-3 or Hyper-IL-6. Proliferation was measured as indicated under “Experimental Procedures.” E, cell surface expression of N-terminally myc-tagged gp130, gp130ΔYY, or gp130ΔD1ΔYY (open histograms) in stably transduced Ba/F3-gp130 and Ba/F3 cells was analyzed by FACS. Ba/F3-gp130 (left panel) and Ba/F3 (center and right panels) cells were used as negative controls (filled histograms).
oncostatin M, or ciliary neurotrophic factor (19). As a control, B-P4 did not inhibit the proliferation of Ba/F3-gp130-gp130ΔYY cells stimulated with IL-3, indicating that B-P4 specifically blocked the activity of gp130ΔYY in Ba/F3-gp130-gp130ΔYY cells (Fig. 4D).

**DISCUSSION**

Constitutive activation of the gp130-dependent transcription factor STAT3 has been implicated in many human neoplastic malignancies, including multiple myeloma (4, 22, 23), prostate cancer, melanoma, ovarian cancer, renal carcinoma (24), as well as gastric cancer (25). Artificially dimerized STAT3 has been shown to exhibit oncogenic potential, and STAT3 was therefore designated as an oncogene (26). The IL-6/gp130 signaling pathway is a candidate for constitutive STAT3 activation in tumors (27). Increased STAT3 phosphorylation was found in IHCAs (2). Interestingly, gp130 gene mutations were found in 60% of the analyzed IHCAs. It turned out that these mutations resulted in ligand-independent dimerization of gp130 receptor chains and constitutive STAT3 phosphorylation. This was the first report on somatic mutation of gp130 in tumors (2), and in combination with the potential to induce cytokine-independent cellular proliferation shown in this study, gp130 can be defined as an oncogene involved in benign human tumors that contributes to the inflammatory phenotype (2).

All mutations of gp130 found in IHCAs were deletions within the cytokine binding interface of domain 2 (2). Here, we analyzed a frequently occurring gp130 mutation (gp130/Y186-Y190, gp130YY) found in four of 26 IHCAs. Six more patients carried mutations from Ser-187-Y190 (gp130SY) that were also covered in Tyr-186 to Tyr-190 (2). We show that gp130ΔYY leads to ligand-independent, long-term proliferation of Ba/F3 cells and constitutive STAT3 phosphorylation. Interestingly, deletion of domain 1 from gp130YY resulted in a signaling-incompetent receptor chain, indicating that domain 1 contributes to ligand-independent receptor activation. However, dimerization of gp130ΔYY was independent of the presence of the D1 domain. The neutralizing anti-gp130 mAb B-T2 directed against D1 did not inhibit receptor activation of gp130ΔYY, indicating that the gp130 homodimerization induced by IL-6/IL-6R is fundamentally different from the homodimerization of gp130ΔYY. Homodimerization of the wild-type gp130 receptor is facilitated by contacts of gp130 CBM (domain 2 and 3) to the binding site II of IL-6 and of gp130 D1 to the binding site III of IL-6, whereas the IL-6R contacts IL-6 via the binding site I (16). We speculate that homodimerization of gp130ΔYY is facilitated by the interaction of the mutated D2 (CBM) of one receptor with the D2 of the other.

![FIGURE 3. No inhibition of ligand-independent proliferation of Ba/F3-gp130-gp130ΔYY by sgp130 or sgp130Fc or the anti-gp130 mAb B-T2. A, equal numbers of Ba/F3-gp130 cells stably transduced with myc-gp130ΔYY were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of B-T2 (0, 0.1, 0.5, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under “Experimental Procedures.” As a control, Ba/F3-gp130-myc-gp130ΔYY cells were treated with 1 ng/ml Hyper-IL-6 plus sgp130. C, equal numbers of Ba/F3-gp130 cells stably transduced with gp130ΔYY-myc were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of sgp130 (0, 0.1, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under “Experimental Procedures.” As a control, Ba/F3-gp130-myc-gp130ΔYY cells were treated with 1 ng/ml Hyper-IL-6 plus sgp130. D, after 6 h of serum starvation, Ba/F3 cells stably transduced with myc-gp130ΔYY or myc-gp130 were stimulated for 5 min with Hyper-IL-6, Hyper-IL-6 plus sgp130Fc, or sgp130Fc or left untreated. STAT3 phosphorylation was analyzed by Western blot analysis.](https://doi.org/10.1074/jbc.R112.377687)
other receptor. However, future studies are needed to fully explore the mechanism of ligand-independent gp130/H9004YY receptor activation.

Moreover, the wild-type gp130 receptor formed stable heterodimers with gp130/H9004YY, and overexpression of the wild-type gp130 receptor blocked constitutive activation of gp130/H9004YY (2). A likely mechanism for this inhibition is that interaction of the extracellular parts of wild-type and mutated gp130 receptors resulted in inactive gp130/gp130/H9004YY heterodimers. Surprisingly, soluble gp130 variants (sgp130 and sgp130Fc) did not inhibit gp130/H9004YY-induced cellular proliferation. This paradoxical situation might be explained by a limited access of sgp130 in the sterical correct orientation to the cell surface bound gp130 protein, which might also explain why sgp130 cannot inhibit gp130/H9004YY-induced cellular proliferation.

However, constitutive ligand-independent activation of gp130/H9004YY was blocked by the neutralizing anti-gp130 mAb B-P4. The epitope of B-P4 is located within the fibronectin type III domain 4 of gp130 (gp130-D4). Truncation of the fibronectin-like type III domains results in gp130 molecules devoid of signaling capacity (28), and it has been speculated that the functional role of the fibronectin type III domains is the assembly of the transmembrane domains in close proximity to allow activation of gp130-associated intracellular JAKs (29). Interestingly, B-P4 has been shown to block only gp130 signaling induced by IL-11 but not by IL-6 or the other members of the IL-6 family, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor (19). Cardiotrophin 1, cardiotrophin-like cytokine, and IL-27 were, however, not investigated so far (19). This might indicate that signaling of gp130/H9004YY mimics IL-11 signaling. IL-11 was shown to promote gastric cancer via gp130 and STAT3 phosphorylation (30). This view is supported by the finding that IL-11 but not IL-6 was overexpressed in IHCAs (2). IL-11 was, however, only overexpressed in IHCAs that did not harbor gp130 mutations (2), suggesting that IHCAs are, to some extent, driven by IL-11 via wild-type gp130. After somatic mutation of gp130 into an IL-11-like constitutively active gp130 variant, the necessity of IL-11-driven gp130 signal transduction might be abrogated, resulting in down-regulation of IL-11 expression levels.

No malignant transformation was found in IHCAs with gp130 mutations, but two of 111 analyzed cases of malignant transformation of IHCAs into hepatocellular carcinoma carried mutations in gp130 and in the β-catenin pathway, suggest-

**FIGURE 4. Biological activity of gp130ΔYY can be suppressed by the neutralizing anti-gp130 mAb B-P4 but not by B-R3.** A, equal numbers of Ba/F3-gp130-gp130/H9004YY-myc cells were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of B-R3 (0, 0.1, 0.5, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under “Experimental Procedures.” As a control, Ba/F3-gp130 cells were treated with 10 ng/ml Hyper-IL-6 and B-R3. B, equal numbers of Ba/F3-gp130-gp130/H9004YY-myc cells were cultured for 3 days in the presence of IL-3 (1 ng/ml) and B-R3 (5 μg/ml). Proliferation was measured as indicated under “Experimental Procedures.” As a control, Ba/F3-gp130 cells were treated with 1 ng/ml Hyper-IL-6 and B-R3 (5 μg/ml). C, equal numbers of Ba/F3-gp130-gp130/H9004YY-myc cells were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of B-P4 (0, 0.1, 0.5, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under “Experimental Procedures.” As a control, Ba/F3-gp130 cells were treated with 10 ng/ml Hyper-IL-6 and B-P4 (10 μg/ml). D, equal numbers of Ba/F3-gp130-gp130/H9004YY-myc cells were cultured for 3 days in the presence of IL-3 (1 ng/ml) and B-P4 (10 μg/ml). Proliferation was measured as indicated under “Experimental Procedures.”
ing a rare interplay of these pathways in malignant transformation (2). In conclusion, blockade of constitutive activation of mutant gp130 by B-P4 might open a possibility to therapeutically block gp130-induced STAT3 phosphorylation in hepatic adenomas and in a subclass of hepatocellular carcinomas.

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