Alternative Intrinsic Polyadenylation Generates the Interleukin-6 Trans-signaling Inhibitor sgp130-E10*

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Jan Sommer†, Christoph Garbers‡, Janina Wolf‡, Ahmad Trad‡, Jens M. Moll†, Markus Sack‡, Rainer Fischer‡, Joachim Grötzinger§, Georg H. Waetzig¶, Doreen M. Floss‡, and Jürgen Scheller‡

From the †Institute of Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, 40225 Düsseldorf, the §Institute of Biochemistry, Medical Faculty, Christian-Albrechts-University, 24118 Kiel, the ‡Institute of Molecular Biotechnology, RWTH Aachen University, 52062 Aachen, and the ¶CONARIS Research Institute AG, 24118 Kiel, Germany

Background: Interleukin (IL)-6 trans-signaling is inhibited by naturally occurring soluble gp130 (sgp130) variants.

Results: Alternative intrinsic polyadenylation in the gp130 gene creates the novel natural IL-6 trans-signaling inhibitor sgp130-E10.

Conclusion: Alternative mRNA polyadenylation contributes to regulation of IL-6 trans-signaling intensity.

Significance: This is the first report on a soluble isoform of the key cytokine co-receptor gp130 originating from alternative polyadenylation.

Interleukin (IL)-6 signals via a receptor complex composed of the signal-transducing β-receptor gp130 and the non-signaling membrane-bound or soluble IL-6 receptor α (IL-6R; sIL-6R), which is referred to as classic and trans-signaling, respectively. IL-6 trans-signaling is functionally associated with the development of chronic inflammatory diseases and cancer. Soluble gp130 (sgp130) variants are natural inhibitors of trans-signaling. Differential splicing yields sgp130 isoforms. Here, we describe that alternative intrinsic polyadenylation in intron 10 of the gp130 transcript results in a novel mRNA coding for an sgp130 protein isoform (sgp130-E10) of 70–80 kDa. The sgp130-E10 protein was expressed in vivo in human peripheral blood mononuclear cells. To assess the biological activity of sgp130-E10, we expressed this variant as Fc-tagged fusion protein (sgp130-E10Fc). Recombinant sgp130-E10Fc binds to a complex of IL-6 and sIL-6R, but not to IL-6 alone, and specifically inhibits IL-6 trans-signaling. Thus, it might play an important role in the regulation of trans-signaling in vivo.

The signaling complex of interleukin (IL)-6 is composed of the signal-transducing β-receptor gp130 and the non-signaling membrane-bound or soluble IL-6 receptor α (IL-6R; sIL-6R), referred to as classic and trans-signaling, respectively. IL-6 binds in a first step to the IL-6R, and this IL-6/sIL-6R complex recruits two gp130 molecules with high affinity, which initiates intracellular signal transduction. Soluble isoforms of gp130 (sgp130) compete with the membrane-bound gp130 for the binding to the complex of IL-6/sIL-6R, thereby specifically antagonizing IL-6 trans-signaling (1). Blocking IL-6 trans-signaling was shown to ameliorate chronic inflammatory diseases (2, 3). Levels of about 400 ng/ml sgp130 are found in normal human serum (4), but its cellular origin and the mechanism of its generation are still poorly understood. At least three soluble gp130 receptor isoforms of ~110, 90, and 50 kDa have been detected in serum or urine (4–6). Only the protein corresponding to the differentially spliced mRNA of the smallest (50 kDa) isoform sgp130-RAPS was verified by Western blotting using specific antibodies directed against the novel C terminus of sgp130-RAPS (5, 7, 8). Two additional differentially spliced gp130 cDNAs were described, but have not been shown to be endogenously translated into sgp130 proteins (7, 8). The expected size of these variants would, if at all, correspond to the largest endogenous sgp130 variant of ~110 kDa (4, 6). It is unknown whether sgp130 variants are also generated by ectodomain shedding of the transmembrane gp130 receptor.

Besides differential splicing, alternative intrinsic polyadenylation creates alternative protein isoforms (9). The use of an intrinsic polyadenylation site (PAS) leads to shortened mature mRNAs and thereby to truncated protein isoforms. If the C-terminal part of a full-length protein is important for its function, intrinsic polyadenylation might abolish or alter the function of the shortened protein isoform. Recently, soluble dominant-negative receptor tyrosine kinase isoforms were identified, which originated from alternative intrinsic polyadenylation (10).

In the present study, we have discovered a novel sgp130 mRNA, which results from alternative intrinsic polyadenylation in intron 10. The resulting isoform (sgp130-E10) consists of the four N-terminal domains of gp130 and ends in the loop region between domains 4 and 5. sgp130-E10 was found to be primarily expressed in peripheral blood mononuclear cells (PBMCs), with a peak expression in CD14+ monocytes.
resulting in a protein of 70–80 kDa. We expressed sgp130-E10 as Myc-His- or Fc-tagged variants and showed that sgp130-E10 specifically inhibits IL-6 trans-signaling, albeit with much lower efficiency than full-length sgp130.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**Hyper-IL-6 dependent Ba/F3-gp130 cells were created and selected as described previously (11). CHO-K1 cells and HEK-293 cells were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig, Germany). Cells were cultured in standard DMEM growth medium consisting of DMEM high glucose medium (Life Technologies/Gibco; Darmstadt, Germany) supplemented with 10% FBS (Life Technologies/Gibco), penicillin (60 mg/liter, Life Technologies/Gibco), and streptomycin (100 mg/liter, Gibco) at 37°C with 5% CO2 in a water-saturated atmosphere. For cultivation of Ba/F3-gp130 cells, the standard DMEM growth medium was additionally 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 (12, 13). It was also used in an Fc-tagged form (Hyper-IL-6Fc). Hyper-IL-6 was expressed and purified as described previously (12). Antibodies against signal transducer and activator of transcription (STAT)-3 and its Tyr705-phosphorylated epitope were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). The antibody directed against Myc tag was expressed and purified as described previously (14). The antibody directed against human Fc tag and secondary antibodies used for Western blot analyses were purchased from Pierce (Thermo Fisher Scientific). The recombinant protein sgp130Fc was expressed and purified as described previously (1, 15).

**Generation of Hybridoma-producing Monoclonal gp130 E10 Antibodies—**A peptide corresponding to amino acids 440–448 (CDFQGLYL) of the C-terminal region of sgp130-E10 was synthesized (Peptides&Elephants; Potsdam, Germany) and conjugated to KLH and KLH-conjugated peptide emulsified with 20% of adjuvant (GERBU Biotechnik; Heidelberg, Germany). BALB/c mice were immunized intraperitoneally with 50 μl of peptide conjugated to KLH in 60 μl of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4) emulsified with 40 μl of GERBU adjuvant MM (GERBU Biotechnik; Heidelberg, Germany).

The mice were boosted intraperitoneally on days 14 and 21 with 100 μg of KLH-conjugated peptide emulsified with 20% of the adjuvant. The last two doses (100 μg) were performed on days 28 and 29 without adjuvant. The hybridoma fusion was performed on day 30; spleen cells from immunized animals were collected and fused with Ag8.653 myeloma cells using a pcDNA3.1 vector (Life Technologies) containing either a Myc-His or an Fc tag.

**Transfection and Selection of Stably Transfected CHO-K1 Cells—**CHO-K1 cells were transiently transfected using TurboFect™ according to the manufacturer’s instructions (Thermo Fisher Scientific Biosciences). The transfection efficiency was typically ~70%, which was estimated by microscopic analysis (Keyence, BZ-9000) of green fluorescent protein expression 24 h after transfection. Transfected cells were cultured in standard DMEM growth medium; from 48 h after transfection onwards, transfected cells were selected in 1 mg/ml G-418 sulfate (Genaxxon Biosciences; Ulm, Germany) for at least 1 week. After 1 week of antibiotic selection, the remaining cells were detached with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in PBS and transferred to a new 10-cm cell culture dish. Cells were then cultured in standard DMEM growth medium with 1 mg/ml G-418 sulfate for another 4 days. Subsequently, cells were detached with trypsin/EDTA as described above and transferred to a 96-well plate at a theoretical concentration of 0.1 cells/well. After seeding, cells were cultured in standard DMEM growth medium with 1 mg/ml G-418 sulfate for another 9 days. To identify wells with single cell clones, all wells were initially and repeatedly checked by microscopy. Supernatants of each single-clone well were analyzed for sgp130-E10Fc or sgp130-E10Myc-His secretion by ELISA (Duolset, DY228; R&D Systems; Wiesbaden, Germany). The cell clone with the highest concentration of sgp130-E10Fc or sgp130-E10Myc-His, respectively, in the supernatant was chosen for further cell expansion and protein production.

**Proliferation Assay—**Ba/F3-gp130 cells were washed three times with sterile PBS and suspended in standard DMEM growth medium without Hyper-IL-6 at 5 × 105 cells/well of a 96-well plate. The cells were cultured for 3 days in a final volume of 100 μl with additional 10 ng/ml Hyper-IL-6 and sgp130 variants as indicated under “Results.” The CellTiter-Blue cell viability assay (Promega; Mannheim, Germany) was used to determine the amount of viable cells following the manufacturer’s instructions and measured on a Tecan Infinite 200 pro fluorometer (excitation wavelength, 560 nm/9 nm; emission wavelength, 590 nm/20 nm; gain, 90; software, Tecan i-control, TECAN, Crailsheim, Germany). All values were measured in triplicate.

**Coomassie Blue Staining—**SDS-PAGE gels were incubated for 1 h in Coomassie Blue stain (water supplemented with 0.1% Coomassie R250, 10% acetic acid, and 40% methanol) under gentle agitation. Coomassie Blue stain was replaced by destaining solution (water supplemented with 20% methanol and 10% acetic acid) and kept under gentle agitation. Destaining solu-
tion was exchanged several times, until the background of the gel stain was minimized.

Western Blotting—For Western blotting, proteins separated by SDS-PAGE were transferred onto PVDF membranes (Roti®-PVDF; Carl Roth; Karlsruhe, Germany) by semidry electroblotting. Membranes were blocked in a solution of Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.6 and 150 mM NaCl) supplemented with 0.05% Tween 20 and 5% (w/v) skimmed milk powder, and probed overnight with the indicated antibodies at 4 °C followed by incubation at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific/Perbio Science). Immunoreactive proteins were detected using a chemiluminescence kit (Amersham Biosciences™ ECL™ Prime Western blotting detection reagent; GE Healthcare; Freiburg, Germany) following the manufacturer's instructions.

Purification of Recombinant sgp130-E10Fc—2 × 10^6 cells of the selected CHO-K1 cell clone secreting sgp130-E10Fc into the cell culture supernatant were seeded into a 225-cm² cell culture flask containing 110 ml of standard DMEM growth medium. After 6 days, the supernatant was harvested and centrifuged at 3,200 × g and 4 °C for 100 min. The resulting supernatant was filtered (bottle top filter, 0.22-μm pore diameter; Nalgene; Rochester, NY) and stored at −80 °C. Before chromatography, the supernatant was thawed, and its pH was adjusted to 6.6. The supernatant was loaded on a protein-A column (HiTrap protein A HP; GE Healthcare) at a flow rate of 3 ml/min. The column was washed with 30 column volumes of PBS. sgp130-E10Fc protein was eluted with a 3:7 mixture of 50 mM citric acid buffers adjusted to pH 5.5 and 3.25, respectively, at a flow rate of 1 ml/min. Fractions of 1 ml were collected. Fractions containing the protein peak were pooled, and the pH was adjusted to 8.0. The protein was further purified by size exclusion chromatography (see below).

Purification of Recombinant sgp130-E10Myc-His—Cell culture supernatant containing sgp130-E10Myc-His was produced as described for sgp130-E10Fc. The pH of the supernatant was adjusted to 8.0, and the protein was purified by affinity chromatography using a HisTrap™ Excel column (GE Healthcare) according to the manufacturer's instructions. The protein was further purified by size exclusion chromatography (see below).

Size Exclusion Chromatography—The sgp130-E10Fc protein and the sgp130-E10Myc-His protein were further purified on a Superdex™ 750 10/300 GL column (GE Healthcare) using PBS as the mobile phase with a constant flow rate of 1 ml/min. Fractions of 1 ml were collected and analyzed by SDS-PAGE or ELISA. sgp130-E10 protein-containing fractions were pooled, concentrated, and analyzed by SDS-PAGE, Western blotting, and ELISA.

Protein Concentration Measurements—The concentration of sgp130-E10 proteins was determined by ELISA (Human sgp130 DuoSet, R&D Systems) following the manufacturer's instructions.

Immunoprecipitation—Purified sgp130-E10Fc protein or conditioned HEK-293 cell medium containing sgp130Fc, sgp130-E10Fc, or sgp130-E10Myc-His after transient transfection was incubated with Hyper-IL-6- or Hyper-IL-6Fc-containing medium overnight at 4 °C followed by the addition of 50 μl of protein A-Sepharose (Pierce® protein A-Agarose, 50% slurry; Thermo Fisher Scientific) for at least 4 h at 4 °C. Immunoprecipitates were washed five times with washing buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5 mM EDTA, plus one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science; Mannheim, Germany) in 50 ml of buffer). 100 μl of Laemmli sample buffer (5 × Laemmli buffer stock solution: 500 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.03% bromophenol blue) were added to the Sepharose and incubated at 95 °C for 10 min.

Binding Assay—Microtiter plates (F96 MaxiSorp NUNC-Immuno plate; Thermo Fisher Scientific) were coated with Hyper-IL-6 (5 μg/ml) in PBS or sgp130-E10Fc (5 μg/ml) in PBS overnight at room temperature. After blocking with 1% (w/v) BSA in PBS for 2 h, 50-μl aliquots of purified sgp130-E10Fc, sgp130-E10Myc-His, or sgp130Fc (50 pm to 100 nm) or IL-6 (0–1 μg/ml) were added. The plate was incubated for 1 h at room temperature. sgp130 variants bound to the plate were detected by the biotinylated detection antibody of the gp130 ELISA used for determination of gp130 concentration (see above) following the manufacturer’s instructions. IL-6 bound to the plate was detected by the biotinylated IL-6 antibody of the human interleukin-6 ELISA kit (ImmuNoTools; Friesoythe, Germany) following the manufacturer’s instructions.

Competition Assay—Microwell plates (F96 MaxiSorp NUNC-Immuno plate, Thermo Fisher Scientific) were coated with sgp130Fc (5 μg/ml) in PBS and incubated overnight at room temperature. After blocking with 1% (w/v) BSA in PBS for 2 h, 50-μl aliquots of Hyper-IL-6 (1 μg/ml) with purified sgp130-E10Fc or sgp130Fc (50 pm to 100 nm) were added. The plate was incubated for 1 h at room temperature. Hyper-IL-6 bound to the plate was detected by the biotinylated IL-6 antibody of the human interleukin-6 ELISA kit (ImmuNoTools) following the manufacturer’s instructions.

Stability Assay—For investigating the stability of sgp130-E10Fc in comparison with sgp130Fc, both proteins were added to standard DMEM growth medium consisting of DMEM high glucose medium supplemented with 10% FBS, penicillin (60 mg/liter), and streptomycin (100 mg/liter) with or without 10 ng/ml Hyper-IL-6 and incubated at 37 °C for 72 h in tubes. Every 24 h, a sample of 200 μl was taken and stored at −80 °C. Finally, all samples were analyzed by gp130 ELISA following the manufacturer’s instructions (see above). The concentrations at 0 h were set to 100%, and the relative concentrations for later time points were calculated.

ELISA—To detect sgp130 serum levels, we used the human sgp130 DuoSet (R&D Systems) according to the manufacturer's instructions. To specifically detect sgp130-E10, we used E10/1 as capture antibody (final concentration 4 μg/ml in PBS) and performed all other steps according to the manufacturer’s instructions for the human sgp130 DuoSet. Serum from healthy humans was obtained as described previously (18). Ethic approval for sgp130 determination in the serum of healthy volunteers for this study was obtained from the Institutional Review Board of the Heinrich–Heine-University.

Phospho-STAT3 Assay—For detection of phospho-STAT3, Ba/F3-gp130 cells were washed three times with sterile PBS and
starved for 4 h in serum-free DMEM before adding 10 ng/ml Hyper-IL-6 and/or sgp130 variants for 15 min at 37 °C as indicated under "Results." Hyper-IL-6 was preincubated with sgp130 variants for 30 min at 37 °C before adding to the cells. Subsequently, cells were centrifuged, and the cell pellet was directly heated in 100 μl of Laemmli sample buffer at 95 °C for 10 min followed by Western blotting as described above.

Sequence Alignment—Alignment of the intron 10 sequences of gp130 from different animal species was performed using the Clustal Omega software.

Isolation of PBMCs—For the isolation of RNA, PBMCs were isolated from total animal blood samples or from the Buffy coat of nine healthy blood donors using LSM 1077 lymphocyte separation medium (PAA Laboratories; Cölbe, Germany) following the manufacturer’s instructions. Cells were immediately frozen at −80 °C until RNA isolation.

For the detection of sgp130 protein forms in PBMCs, 35 ml of anti-coagulated blood were loaded onto 15 ml of Histopaque 1077 (Sigma-Aldrich). After centrifugation at 400 × g for 30 min at room temperature without braking, the layer containing the PBMCs was isolated and washed 4–5 times with PBS supplemented with 2 mM EDTA. For immunodetection of different forms of sgp130, 2.5 × 10^6 cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Triton X-100, cOmplete protease inhibitor mixture (Roche Applied Science)). After separation of proteins via 10% SDS-PAGE, sgp130 variants were detected using the B-R3 gp130 antibody (Abcam; Cambridge, UK) and the antibody E10/1 (see above; hybridoma supernatant diluted 1:200) for sgp130-E10 followed by mouse-POD (Thermo Fisher Scientific) as a secondary antibody. Protein bands were visualized by SuperSignal® West Femto maximum sensitivity substrate (Thermo Fisher Scientific).

RNA, cDNA, and RT-PCR—RNA was extracted from the cells using the peqGOLD total RNA kit (S-Line) (PEQLAB Biotechnologie; Erlangen, Germany) following the manufacturer’s instructions, and 5 μg of RNA were used for cDNA synthesis using RevertAid reverse transcriptase (Thermo Fisher Scientific) following the manufacturer’s instructions and using the oligo(dT) method. PCR reactions on cDNA templates were performed using Dream Taq DNA polymerase (Thermo Fisher Scientific) following the manufacturer’s instructions and using adapted PCR protocols and primers. For amplifying sgp130-E10 cDNA from human, dog, and pig samples, we used primers annealing to exon 9 and intron 10. As a control, primers annealing to exon 9 and 11 were used to amplify all gp130 transcripts including also any sgp130 transcripts except for sgp130-RAPS and sgp130-E10. For mouse cDNA, primers annealing to exon 8 and intron 10 were used to amplify sgp130-E10, and primers annealing to exon 8 and exon 10 were used to amplify total gp130 as a control.

Oligonucleotides—Primers for the human sgp130-E10 isoform (5'-TACAGAAGTGTACAATCTGTGGTG-3' and 5'-CAAGAGTCGGCTCCATCTCACAG-3') and all human gp130 transcripts except for sgp130-RAPS (5'-TACAGAATGTACAATCTGTGGTG-3' and 5'-CTGTGCCATCAGCATATACTGGAG-3') were used in PCR analysis. The sequences of the oligonucleotides used for amplification of gp130 transcripts of other mammals will be provided upon request.

Quantitative RT-PCR (qPCR)—Using SYBR® Green PCR master mix (Life Technologies/Applied Biosystems), the qPCR reaction was set up in triplicates using 25 ng of cDNA as a template. The relative expression levels of sgp130-E10 were calculated for each cell line and condition and also for primary cells after normalization to the respective gp130 levels. Student’s t test was used for statistical analysis of the difference in expression between gp130 and sgp130-E10.

Surface Plasmon Resonance Spectroscopy—Experiments were performed on a BiacoreT200 Instrument (GE Healthcare) using PBS supplemented with 0.05% Tween 20 as running buffer. A human Fc capture antibody was immobilized to the surface of a CM5 sensor chip according to the manufacturer’s instructions. Binding experiments were performed at 25 °C and a flow rate of 30 μl/min. For getting comparable binding signals, sgp130Fc and sgp130-E10Fc were diluted to give capture levels of 72 RU and 144 RU, respectively. Hyper-IL-6 samples were injected at concentrations from 0.594 to 151 nM using a 2-fold series dilution. The contact time of the association phase was 180 s followed by dissociation for 600 s. The high affinity component was evaluated by globally fitting the dissociation rate in the 400–600 s time window of the dissociation phase. The association rate was then determined for the two lowest concentrations. The binding curves simulated for the simple monovalent binding of the high affinity component were laid over the measured binding curve to illustrate where deviation occurs.

RESULTS

Identification of a Soluble gp130 Receptor mRNA Originating from Alternative Polyadenylation—Initially, we searched for alternative PAS in the introns of human gp130. A potential alternative PAS was identified in intron 10, which was accompanied by a premature stop codon in the potential sgp130-E10 transcript (Fig. 1A). Subsequently, we verified the transcription of sgp130-E10 by RT-PCR with primers spanning exon 9 to intron 10 and thereby excluding amplification of genomic DNA. A PCR product with the expected size of 271 bp was observed in all human cell lines tested (Fig. 1B). Amplification of gp130 using primers binding in exon 9 and exon 11 with an expected amplicon size of 485 bp was used as internal control (Fig. 1B).

By performing a PCR primer walk in intron 10, we indirectly showed that the alternative PAS was used for alternative polyadenylation. All primers for the distances −890 to −12 resulted in the expected PCR product sizes, in contrast to the primer that annealed 3' of the potential alternative PAS (Fig. 1C), strongly indicating that the predicted alternative polyadenylation site is used for the transcript. qPCR revealed that about 2% of the total gp130 transcripts in the liver carcinoma cell lines HepG2 and Hep3B as well as in the human embryonic kidney cell line HEK-293 represented sgp130-E10. Amplification of total gp130 transcripts included membrane-bound and soluble gp130 transcripts generated by differential splicing without sgp130-RAPS and sgp130-E10. In the MCF-7 mammary carcinoma and Jurkat T cell lymphoma cell lines, the percentage of sgp130-E10 was about 7 and 60% of the total gp130 transcripts, respectively (Fig. 1D). This indicates that the expression of
Novel gp130 Isoform by Alternative Polyadenylation

(A) Diagram showing the alternative polyadenylation sites in gp130 isoforms.

(B) Gel electrophoresis showing the bands for gp130 and sgp130-E10.

(C) Image showing the distance of reverse primer to alternative poly(A) site in bp.

(D) Graph showing the mRNA level (fold increase) for gp130 and sgp130-E10 in different cell lines.

(E) Graph showing the mRNA level (fold increase) for gp130 and sgp130-E10 in PBMCs and mammary carcinoma cell lines.

(F) Table showing the polyadenylation sites in different species.

(G) Image showing the band patterns for gp130 and sgp130-E10 in different species.
Novel gp130 Isoform by Alternative Polyadenylation

sgp130-E10 is higher in cells in which IL-6 trans-signaling is important for maintenance of the inflammatory state. Thus, sgp130-E10 might be a natural inhibitor of trans-signaling produced directly by pro-inflammatory cells at the site of inflammation to limit their own activation. To verify this finding, we investigated the expression of sgp130-E10 in five other mammalian carcinoma cell lines (SKBR3, BT20, T47D, NCF12A, and Cal85-1) as well as in peripheral blood mononuclear cells (PBMCs). All mammary carcinoma cell lines had a similar expression of sgp130-E10 of about 4% of the total gp130 transcripts (Fig. 1E). In contrast, the percentage of sgp130-E10 was ~21% of all gp130 transcripts in PBMCs (Fig. 1E).

Expression of the sgp130 variant identified by Diamant et al. (8) was very low because the qPCR signals were close to the negative control. We have not analyzed the expression of sgp130-RAPS.

An alignment of gp130-intron 10 sequences from different species revealed that the alternative PAS is located at the same position in humans, dogs, monkeys, and cats. Horses and pigs, however, feature potential polyadenylation sites at different locations in intron 10, whereas mice and rats do not have a canonical alternative PAS in intron 10. Moreover, transcripts from all examined species would feature a premature stop codon in the sgp130-E10 transcript with the exception of rats (Fig. 1F). To exemplarily examine mRNA expression of sgp130-E10 in other species, we isolated total RNA from PBMCs of dogs and pigs or spleen cells of mice. As predicted, RT-PCR detected sgp130-E10 transcripts in the dog and pig samples, but not in the murine samples (Fig. 1G). Again, amplification of sgp130-E10 was performed using primers for exon 9 and intron 11. PCR with primers for exon 9 and exon 10 was used as an internal gp130 amplification control (Fig. 1G). In conclusion, we identified a novel sgp130 transcript variant generated by alternative intronic polyadenylation that shows tissue-specific expression patterns.

sgp130-E10 protein Binds to IL-6/Soluble IL-6R (Hyper-IL-6), but Not to IL-6 Alone—Full-length gp130 consists of six extracellular domains (D1–D6), a transmembrane domain, and an intracellular domain (8). For sgp130-E10, exons 1 and 2 encode the 5′-untranslated region, exon 3 encodes the signal peptide, exons 4–8 encode the extracellular domains D1 to D3, and exons 9 and 10 encode D4 followed by four additional amino acids (GLYL) and the premature stop codon encoded by intron 10.

Binding of IL-6/sIL-6R to sgp130 is mediated by D1 (Ig-like domain) and D2/D3 (cytokine-binding module, CBM). We modeled sgp130-E10 in complex with IL-6/sIL-6R and concluded that sgp130-E10 should be able to inhibit IL-6 trans-signaling (Fig. 2A).

The sgp130-E10 cDNA was subcloned into a eukaryotic expression plasmid with either a C-terminal Myc and His tag or an Fc tag from a human IgG1 antibody. cDNAs coding for sgp130-E10Fc or sgp130Fc were transiently transfected into HEK-293 cells. sgp130Fc consists of the extracellular domains D1–D6 of gp130 fused to the Fc-part of a human IgG1 antibody. The Fc fusion results in a constant dimerization of sgp130 and, thus, in a 10–100-fold increase in binding efficiency to IL-6/sIL-6R complex as compared with monomeric sgp130 (1).

The resulting monomeric protein sgp130-E10Myc-His had an apparent molecular mass of 80–90 kDa (Fig. 2B). sgp130-E10Myc-His was precipitated from conditioned HEK-293 cell culture supernatants by Hyper-IL-6Fc (Fc-tagged Hyper-IL-6, a fusion protein of IL-6 and sIL-6R) in combination with protein A-agarose beads (Fig. 2B). In addition, conditioned HEK-293 cell culture supernatants containing sgp130-E10Fc or sgp130Fc were used to precipitate Hyper-IL-6 via protein A-agarose beads (Fig. 2C). Our results showed that sgp130-E10 in both a monomeric and a dimeric form is able to bind to the IL-6/sIL-6R complex represented by Hyper-IL-6.

Subsequently, stable CHO-K1 cell clones expressing sgp130-E10Myc-His or sgp130-E10Fc were generated and selected for high level expression by ELISA. Recombinant protein expression was determined by ELISA to be about 300 μg/liter supernatant for sgp130-E10Myc-His and 800 μg/liter for sgp130-E10Fc.

The final yield of pure protein was, however, much lower, namely about 1 μg/liter for sgp130-E10Myc-His and ~40 μg/liter for sgp130-E10Fc, which was due to instability of the recombinant proteins and loss of recombinant proteins during the purification via His tag or Fc tag. For this reason, purification of...
sufficient amounts of sgp130-E10Myc-His for further experiments was not possible.

sgp130-E10Fc was purified by affinity chromatography and size exclusion chromatography. The purity and identity of sgp130-E10Fc was verified by Coomassie Blue staining of SDS-PAGE gels (Fig. 3A) and Western blotting (Fig. 3B). Just like sgp130Fc, purified sgp130-E10Fc was able to precipitate Hyper-IL-6 in combination with protein A-agarose beads, indicating that the purified sgp130-E10Fc was biologically active (Fig. 3C).

We performed an ELISA binding assay with Hyper-IL-6-coated plates and free sgp130Fc and sgp130-E10Fc to characterize binding of the sgp130 variants to Hyper-IL-6. In this assay, sgp130Fc and sgp130-E10Fc showed similar binding
Binding constants and affinities of Hyper-IL-6 to sgp130-E10Fc and sgp130Fc

| Ligand         | $R_{\text{capture}}$ | $R_{\text{max}}$ | $k_\text{on}$ | $k_\text{off}$ | $K_D$ |
|---------------|----------------------|-------------------|---------------|----------------|-------|
| sgp130Fc      | 72 s                 | 337 s             | $3.1 \times 10^6$ | $3.1 \times 10^{-5}$ | 9 pM  |
| sgp130-E10Fc  | 144 s               | 25 s              | $1.3 \times 10^6$ | $9.0 \times 10^{-5}$ | 71 pM |

10$^6$ $\text{M}^{-1}$ s$^{-1}$ and dissociates three times more rapidly from (9.0 $\times$ 10$^{-5}$ s$^{-1}$ versus $3.1 \times 10^{-5}$ s$^{-1}$) sgp130-E10Fc than from sgp130Fc. This results in overall apparent affinities of $K_D = 9$ pM for sgp130Fc and of $K_D = 71$ pM for sgp130-E10Fc. The $K_D$ of sgp130Fc to Hyper-IL-6 was in the range of previously described values (15).

Generation of an sgp130-E10-specific Antibody and Detection of sgp130-E10 in PBMCs—Using the synthetic peptide CDFQGLYL deduced from the unique C terminus of sgp130-E10, we generated a monoclonal antibody in mice specifically detecting this sequence. One resulting mAb, E10/1, was tested in Western blots for specific detection of sgp130-E10 using recombinant sgp130-E10Fc, and sgp130Fc served as negative control. E10/1 was able to detect sgp130-E10Fc with a detection limit of ~10 ng and without cross-reactivity to sgp130Fc (Fig. 5A).

We observed the highest mRNA level of sgp130-E10 in PBMCs. Therefore, PBMCs were analyzed for sgp130-E10 expression using the E10/1 mAb. As depicted in Fig. 5B, E10/1 detected a protein with an apparent molecular mass of 70–80 kDa in PBMCs, which corresponded to the expected size of sgp130-E10. The same protein was detected using the commercial gp130 mAb BR-3, albeit with a much lower efficacy. BR-3 specifically detects the extracellular domain D2 of gp130. Next, we used this novel antibody to establish an ELISA that specifically detects sgp130-E10, but not other sgp130 variants. As shown in Fig. 5C, E10/1 was able to capture sgp130-E10Fc in a dose-dependent manner, but not sgp130Fc. sgp130Fc is found in high concentrations in human serum of about 200–400 ng/ml (Fig. 5D), and we therefore asked whether sgp130-E10 contributes to these levels. Indeed, we were able to detect sgp130-E10 in the range of 1 ng/ml (1,713, 1,331, and 644 ng/ml) in 3 out of 8 serum samples from healthy donors (Fig. 5E). In conclusion, our data indicate that sgp130-E10 is translated into a protein in PBMCs, is the dominant sgp130 variant in these cells, and contributes to the sgp130 serum levels in healthy humans.

Biological Activity and Stability of sgp130-E10Fc—To judge the potential of sgp130-E10Fc as a drug candidate as compared with the "gold standard" sgp130Fc, we performed a competitive ELISA to compare the binding properties of sgp130Fc and sgp130-E10Fc to Hyper-IL-6. sgp130Fc was immobilized on a 96-well plate and incubated with saturating concentrations of Hyper-IL-6. Hyper-IL-6 was detected using labeled IL-6 mAbs. As expected, free sgp130Fc or sgp130-E10Fc reduced the detection signal to the baseline. E10/1 was able to capture sgp130-E10Fc in a dose-dependent manner, but not sgp130Fc. sgp130Fc is found in high concentrations in human serum of about 200–400 ng/ml (Fig. 5D), and we therefore asked whether sgp130-E10 contributes to these levels. Indeed, we were able to detect sgp130-E10 in the range of 1 ng/ml (1,713, 1,331, and 644 ng/ml) in 3 out of 8 serum samples from healthy donors (Fig. 5E). In conclusion, our data indicate that sgp130-E10 is translated into a protein in PBMCs, is the dominant sgp130 variant in these cells, and contributes to the sgp130 serum levels in healthy humans.
results that sgp130-E10Fc specifically binds to IL-6/sIL-6R, albeit with a 5–10-fold lower efficacy than sgp130Fc.

During protein purification, we observed a reduced stability of sgp130-E10Myc-His and sgp130-E10Fc as compared with sgp130Fc. Therefore, we analyzed the time-dependent stability of the Fc-compounds by ELISA. sgp130-E10Fc and sgp130Fc were incubated with or without Hyper-IL-6 in standard DMEM growth medium at 37 °C. The half-life of sgp130-E10Fc (12 h) was strongly reduced as compared with the half-life of sgp130Fc (72 h). Stability of both sgp130 variants was independent of the presence of Hyper-IL-6 (Fig. 6B).

Subsequently, we investigated whether sgp130-E10Fc was able to inhibit IL-6/sIL-6R-induced cellular proliferation and STAT3 phosphorylation. We used Ba/F3 cells stably transduced with human gp130, which proliferate in the presence of Hyper-IL-6 in a phospho-STAT3-dependent manner (11), as a model system. sgp130-E10Fc and sgp130Fc inhibited Ba/F3-gp130 cell proliferation in a dose-dependent manner. From the data of these experiments, we calculated an IC_{50} of 67 nM for sgp130-E10Fc (Fig. 6D) and concluded that, although sgp130-E10Fc does inhibit IL-6 trans-signaling, it is about 1,000-fold less active than sgp130Fc with an IC_{50} of 0.067 nM in this system.

FIGURE 5. Specific detection of sgp130-E10 by the monoclonal sgp130-E10 antibody E10/1 in PBMCs and serum. A, Western blot (WB) analysis of sgp130-E10Fc and sgp130Fc in different concentrations. The monoclonal sgp130-E10 antibody E10/1 specifically detected recombinant sgp130-E10Fc with a detection limit of ~10 ng; E10/1 did not detect sgp130Fc. B, Western blot (WB) analysis of the sgp130-E10 isoform in human PBMC lysates using the gp130 antibody B-R3 and the novel sgp130-E10 antibody E10/1. sgp130-E10 was specifically detected at the expected apparent molecular mass of 70–80 kDa. C, ELISA with the sgp130-E10 antibody E10/1 as capture antibody, demonstrating the specific detection of sgp130-E10Fc, but not sgp130Fc. D and E, sgp130 (D) or sgp130-E10 (E) in the sera of eight healthy volunteers was quantified by specific ELISA.

FIGURE 6. Purified sgp130-E10Fc is biologically active, but inferior to sgp130Fc in terms of binding to Hyper-IL-6, activity and stability. A, competitive ELISA with coated sgp130Fc and competition with either sgp130Fc or sgp130-E10Fc for Hyper-IL-6 binding. Standard errors were calculated and indicated. A representative experiment out of two independent experiments is shown. B, stability assay for sgp130Fc and sgp130-E10Fc. After incubation at 37 °C in cell culture medium for the indicated times, samples were analyzed using gp130 ELISA. The starting concentration was arbitrarily set to 100%, and the remaining protein was calculated and plotted. Standard errors were calculated and indicated. Two independent experiments were performed. Mean values of the two experiments were calculated and are indicated. C, Ba/F3-gp130 cell proliferation assay comparing the bioactivities of sgp130-E10Fc and sgp130Fc. Standard errors were calculated and indicated. D, comparison of the inhibitory effect of sgp130-E10Fc or sgp130Fc on phosphorylation of STAT3 (P-STAT3) in Ba/F3-gp130 cells. A representative experiment out of three independent experiments is shown.
Novel gp130 Isoform by Alternative Polyadenylation

VEGFR-2 expression was upregulated almost 300-fold in WI38 cells transfected with the reporter construct compared to the non-transfected control cells. Importantly, this upregulation was not observed in untreated cells. The U1 interferon expression in primary human foreskin fibroblasts was found to be significantly higher in WI38 cells than in the parental HFF cells. The difference in U1 interferon expression between WI38 and HFF cells was the highest among all analyzed cell types. The observed differences in U1 interferon expression specifically observed in WI38 cells suggest that this cell line is more sensitive to interferon induction than the parental HFF cells. The upregulation of VEGFR-2 and U1 interferon expression in WI38 cells transfected with the reporter construct provides evidence for the involvement of alternative polyadenylation in the regulation of these genes.

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