

An Interleukin-6 Receptor-dependent Molecular Switch Mediates Signal Transduction of the IL-27 Cytokine Subunit p28 (IL-30) via a gp130 Protein Receptor Homodimer*[§]

Received for publication, November 1, 2012. Published, JBC Papers in Press, December 3, 2012, DOI 10.1074/jbc.M112.432955

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Background: Anti-inflammatory signaling of IL-27, p28, and EBI3 is mediated by gp130 and Wsx-1.

Results: Signaling of p28 via IL-6R is mediated by a gp130 homodimer.

Conclusion: Signaling of p28 via IL-6R is likely not anti-inflammatory.

Significance: We identify the signal receptor complex of p28/IL-6R.

IL-27 consists of the cytokine subunit p28 and the non-signaling α -receptor EBI3. p28 was shown to additionally act via the non-signaling membrane-bound IL-6 α -receptor (IL-6R) as an agonistic cytokine but also as a gp130 β -receptor antagonist, leading to inhibition of IL-6 signaling. Here, we developed a strategy for bacterial expression, purification, and refolding of murine p28. We show that p28 did not interfere with IL-6- or IL-27-induced signaling, indicating that p28 has no antagonistic properties. Moreover, we demonstrate that murine p28 acts as an agonistic cytokine via the murine and human IL-6R, indicating that p28 exhibits no species specificity. p28 was able to induce p28-trans-signaling via the soluble IL-6R (sIL-6R), a characteristic property that was initially described for trans-signaling of IL-6 via the sIL-6R. Of notice, p28/sIL-6R trans-signaling was inhibited by the IL-6 trans-signaling antagonist, soluble gp130. At higher concentrations, p28 but not IL-6 was able to induce signaling even in the absence of IL-6R or EBI3. Although IL-27 signals via a heterodimer of the β -receptor chains gp130 and Wsx-1, p28/IL-6R specifically recruits two gp130 β -receptor chains for signal transduction. The binding of p28 to a gp130/Wsx-1 heterodimer or a gp130 homodimer is highly selective and controlled by a novel molecular switch induced by EBI3 or IL-6R, respectively.

Cytokines of the IL-6 family, including IL-6, IL-11, IL-27, cardiotrophin-like related cytokine/neurotrophin-1/B-cell stimulating factor 3 (CLC/NNT1/BSF3), leukemia inhibitory

factor, oncostatin M (OSM)³, ciliary neurotrophic factor, cardiotrophin 1 (CT1), and neuropoietin, share the membrane glycoprotein gp130 as a common signal-transducing β -receptor subunit (1, 2). Overlapping biological activities have been reported for this group of cytokines. The functional redundancy of IL-6-family cytokines is due to the usage of the receptor chain gp130, which activates the signal transducer and activator of transcription 1 and 3 (STAT1/3) and the Ras/MAPK signaling pathways (1). IL-6 and IL-11 are the only family members that induce formation of a homodimeric gp130 receptor complex. All other family members signal via gp130-heterodimeric cytokine receptor complexes using Wsx-1, leukemia inhibitory factor receptor, or OSM receptor as a second β -receptor chain. To induce homodimeric gp130-receptor complex formation, IL-6 and IL-11 have to bind to non-signaling IL-6 or IL-11 α -receptor chains (IL-6R or IL-11R), respectively. The IL-6R exists as a membrane-bound and a soluble form (sIL-6R). IL-6 signaling via the membrane-bound IL-6R is called classic signaling and, via the sIL-6R, IL-6 trans-signaling (3). Functionally, mainly proinflammatory activities were assigned to IL-6, e.g. induction of the acute phase response and Th17 differentiation (4).

IL-27 consists of the cytokine subunit p28 and the non-signaling α -receptor Epstein-Barr-virus-induced gene 3 (EBI3). IL-27 exclusively signals via a heterodimer of gp130 and Wsx-1 (5). IL-27 is considered as an anti-inflammatory cytokine and has been shown to be a potent inhibitor of Th17 differentiation (6). Recently, EBI3-independent biological roles for p28 were described. Crabé *et al.* (7) showed that p28 forms a complex with the IL-6R and induces STAT1 and STAT3 signal transduction in IL-27-responsive cells. Controversially, Stumhofer *et al.* (8) showed that p28 did not induce STAT phosphorylation in IL-6- and IL-27-responsive cells but rather acts as an IL-6- and IL-27-cytokine signaling antagonist (8).

* This work was funded by Deutsche Forschungsgemeinschaft (Bonn, Germany) Grant DFG SCHE 907/2-1, SFB877 (A1, A6; SR-J, AC, JG), and by the Research Commission of the Medical Faculty of the Heinrich-Heine-University (to C. G. and J. S.).

[§] This article contains supplemental Figs. S1–S4, Materials and Methods, and References.

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³ The abbreviations used are: OSM, oncostatin M; IL-6R, interleukin-6 receptor; sIL-6R, soluble interleukin-6 receptor; RLU, relative light unit; CBM, cytokine-binding module; CLF, cytokine-like factor-1.

Here, we describe the first protocol for production, refolding, and purification of biologically active murine p28 from bacteria. p28 was able to induce STAT1/3 phosphorylation and cytokine-dependent proliferation of Ba/F3-gp130-IL-6R cells and primary CD4⁺ T cells. Moreover, we show that in opposite to p28/EBI3 (IL-27), p28/IL-6R activates signal transduction solely via the receptor chain gp130 without the need of Wsx-1. p28 can also form a biologically active complex with the sIL-6R, which implies that, like IL-6, p28 trans-signaling is possible on cells lacking membrane-bound IL-6R. Finally, p28 was able to induce signaling on Ba/F3-gp130 cells without the need of the α -receptors IL-6R or EBI3, albeit at higher concentrations.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Ba/F3 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Ba/F3-gp130 cells were from Immunex (Seattle, WA, Ref. 9). Ba/F3-gp130-hIL-6R cells and Ba/F3-gp130-mIL-6R cells have been described previously (10). COS7 and NIH3T3 cells were obtained from the ATCC (LGC Standards, Wesel, Germany). All cells were grown in DMEM high-glucose culture medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% FBS, penicillin (60 mg/l) and streptomycin (100 mg/l) at 37 °C with 5% CO₂ in a water-saturated atmosphere. Ba/F3-gp130 cells were cultured using 10 ng/ml recombinant Hyper-IL-6, which is a fusion protein of IL-6 and the sIL-6R that mimics IL-6 trans-signaling and acts as a growth factor for Ba/F3-gp130 cells (11, 12). Hyper-IL-6 was expressed and purified as described previously. Ba/F3-gp130-hIL-6R cells and Ba/F3-gp130-mIL-6R cells were cultured using 10 ng/ml recombinant human IL-6 instead of Hyper-IL-6. Human IL-6 and soluble human IL-6R were expressed and purified as described previously (13). Murine IL-6 and soluble murine IL-6R were purchased from R&D Systems (Wiesbaden, Germany). The anti-human IL-6R mAb tocilizumab (ACTEMRA/RoACTEMRA) was obtained from Roche. Optimized sgp130Fc was expressed and purified as described previously (14, 15). Anti-phospho-STAT3 mAb (Tyr-705) and anti-STAT3 mAb (124H6) were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). The anti-hIL-6R monoclonal antibody 4-11 was described previously (16). The peroxidase-conjugated secondary antibodies were purchased from Pierce (Thermo Fisher Scientific/Perbio, Bonn, Germany).

Construction of an Escherichia coli Expression Vector for Murine p28—The coding sequence of His-tagged murine p28 lacking the signal peptide (NCBI accession no. NM_145636) was synthesized by Invitrogen/GeneArt (Regensburg, Germany) and subcloned into the *E. coli* expression plasmid pET28a(+) (Novagen/Merck, Darmstadt, Germany).

Expression, Purification, and Renaturation of Murine p28—Expression of p28 was performed in *E. coli* BL21 (DE3) (Merck) (250 ml of culture volume, 37 °C, 1 mM isopropyl 1-thio- β -D-galactopyranoside, 4 h). The pelleted bacterial cells were lysed by sonification (Sonopuls HD2200, Bandelin, Berlin, Germany). p28 inclusion bodies were purified by repeated washing and sonification steps (three times) in 50 mM Tris-HCl (pH 8.0) plus 0.2% Tween 20, followed by three washing steps with 50 mM Tris-HCl (pH 8.0). Purified inclusion bodies were solved in 6 M

guanidine hydrochloride plus 50 mM Tris-HCl (pH 8.0). Denatured p28 was refolded at a protein concentration of 0.1 mg/ml by dialysis against 50 mM acetic acid/acetate (pH 4.0) plus 300 mM sodium chloride. Dialyzed p28 was centrifuged for 30 min at 40,000 $\times g$ at 4 °C. The p28-containing solution was sterile-filtered and concentrated with an Amicon-stirred ultrafiltration cell equipped with a YM10 filter membrane (molecular weight cut off 10,000; Merck Millipore, Schwalbach, Germany). Subsequently, p28 was purified by size-exclusion chromatography using an ÄKTAexplorer chromatography system and equipped with a HiLoad 16/60 Superdex 200 prep grade column (both from GE Healthcare). The fraction containing monomeric p28 was concentrated by a Vivaspin 20 column, molecular weight cut off 10,000 (GE Healthcare, Munich, Germany). Purified p28 protein was analyzed by Coomassie Blue staining and Western blotting after standard SDS-PAGE under reducing conditions. For Western blotting, a biotinylated anti-mouse IL-27 p28 antibody (R&D Systems) and an anti-His-Tag (27E8) mouse antibody (Cell Signaling Technology) were used. For a detailed protocol of Western blotting, see below. Folding of pure monomeric p28 was verified by circular dichroism spectroscopy in a quartz cuvette (Helma, Mühlheim, Germany) with a J-720 CD spectropolarimeter (Jasco, Tokyo, Japan).

Construction of the Murine p28/Human IL-6R Fusion Protein—To assemble the fusion protein of murine p28 and human sIL-6R, the coding sequence of murine p28 was amplified by PCR using pcEP-Pu-IL27 as a template (17). Restriction sites for AgeI (5') and NotI plus SacI (3') were inserted (5' primer, GATCACCGGTGGGCTTCCCAACAGACCC; 3' primer, GATCGAGCTCGCGCCGCTTAGGAATCCCAGGCTGAGCC). The purified PCR product was digested with AgeI and SacI and ligated into pcR-Script-Hyper-IL-6 resulting in a fusion protein of the extracellular part of the human sIL-6R fused via a flexible peptide linker (RGGGGSGGGGSVEPV) to murine p28. The coding sequence was finally subcloned into pcDNA3.1 using KpnI and NotI for digestion.

Transfection of COS7 and NIH3T3 Cells— 5×10^5 COS7 cells or NIH3T3 cells were seeded on 10-cm culture dishes 24 h prior to transfection. Cells were transiently transfected with expression plasmids using Turbofect (Thermo Fisher Scientific/Fermentas, St. Leon-Rot, Germany) according to the instructions of the manufacturer. After 48 h, cells were stimulated with the indicated cytokines, or supernatants were collected and sterile-filtered. Cells were lysed, and Western blot analyses were performed.

Proliferation Assays—Ba/F3 cells were washed three times with sterile PBS and suspended in DMEM containing 10% FBS at a final concentration of 5×10^3 cells/well in a 96-well plate. Cells were incubated with the indicated cytokines and cytokine receptors for 2 days in a final volume of 100 μ l. For inhibitory studies, IL-6R- or gp130-targeting antibodies or sgp130Fc were added as indicated. After 2 days, cell growth was measured using the Cell Titer Blue Cell viability assay (Promega, Karlsruhe, Germany) according to the protocol of the manufacturer. The extinction was measured using a Tecan infinite M200 PRO reader (excitation 560 nm, emission 590 nm, gain 90, i-control 1.7 software, Tecan AG, Crailsheim, Germany). Normalization of relative light units (RLU) was achieved by

subtraction of negative control values. In each experiment, all values were measured in triplicates.

Western Blot Analysis—For detection of protein overexpression, cells were lysed in 250 μ l of mild lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 plus complete protease inhibitor mixture tablet (Roche)). The protein concentration of the lysates was determined using the BCA protein assay kit (Thermo Fisher Scientific) according to the instructions of the manufacturer. 50 μ g of total protein were separated by SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane (GE Healthcare). After blocking with 5% skim milk powder in Tris-buffered saline with Tween 20 (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Tween 20) for 1 h at room temperature, the membrane was probed with a primary antibody against IL-6R (4–11) at 4 °C overnight, and an anti-mouse-IgG antibody was conjugated to horseradish peroxidase. For chemiluminescence detection, the ECL system (GE Healthcare) was used according to the instructions of the manufacturer.

For analysis of STAT3 phosphorylation, Ba/F3-gp130 cells were washed three times with sterile PBS and starved for 6 h in serum-free DMEM. Cells were then stimulated for 15 min at 37 °C with COS7-conditioned supernatants and subsequently lysed in 50 μ l of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 , 1% IGEPAL, Nonidet P-40, 1% Triton-X-100, and complete protease inhibitor mixture tablet). SDS-PAGE and Western blotting were performed as described above. As primary antibodies, monoclonal anti-phospho-STAT3 and, subsequently, anti-STAT3 (both from Cell Signaling Technology) were used. Before probing for non-phosphorylated STAT3, membranes were stripped with stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% β -mercaptoethanol) for 30 min at 65 °C, washed three times in Tris-buffered saline with Tween-20, and blocked again.

Isolation of CD4⁺ T Cells and FACS Analysis—C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Sulzfeld, Germany), Wsx-1-deficient mice (18) were provided by Chris Saris (Amgen, Thousand Oaks, CA). CD4⁺ T cells were isolated from total mouse spleens using CD4 (L3T4) MicroBeads and magnetic cell separation LS columns (both from Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. Cells were then stimulated with either IL-6 (10 ng/ml), Hyper-IL-6 (10 ng/ml), IL-27 (10 ng/ml), or p28 (200 ng/ml) for 15 min. For intracellular staining of phosphorylated tyrosine residues of STAT3, murine T cells were fixed in 2% (w/v) paraformaldehyde at 37 °C for 15 min, followed by permeabilization of cells in 90% (v/v) methanol for 30 min on ice. The cells were subsequently stained for both phosphorylated STAT3 (clone 4/P-STAT3) and total STAT3 antibodies from BD Biosciences. Flow cytometry was performed on cells using a FACSCantoII (BD Biosciences), and data were analyzed using the FACSDiva software.

Surface Plasmon Resonance Measurements—A BiacoreX100 (GE Healthcare) was employed for affinity measurements using a CM5 biosensor chip (GE Healthcare). sgp130Fc (50 mg/ml in 10 mM acetate buffer (pH 4.5)) was immobilized according to the instructions of the manufacturer. p28 was analyzed in a range from 584–4678 nM using 10 mM HEPES (pH 7.4) containing

150 mM NaCl, 3 mM EDTA, 0.05%, and surfactant P20 as running buffer.

Statistical Analysis—Data are expressed as mean \pm S.D. All experiments were performed at least three times independently unless stated otherwise. Statistical analysis was performed using Student's unpaired *t* test. *p* < 0.05 was considered statistically significant. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

RESULTS

p28 Has Agonistic but No Antagonistic Properties—Bacterial expression of p28, which has not been described to date, is an attractive alternative to obtain p28 protein for *in vitro* and *in vivo* studies and is the prerequisite for the determination of the three-dimensional structure. We cloned the cDNA coding for murine p28 lacking the signal peptide into an *E. coli* expression plasmid. Murine p28 was expressed in *E. coli* (supplemental Fig. 1A), purified from inclusion bodies and refolded (supplemental Fig. 1, B and C). Finally, monomeric murine p28 protein was obtained after size-exclusion chromatography. Purity and α -helical folding of p28 was verified by SDS-PAGE followed by Coomassie staining (Fig. 1A), Western blotting (supplemental Fig. 1, D and E), and CD spectroscopy (Fig. 1B), respectively. The final yield of monomeric p28 was about 2.3 mg/liter bacterial culture volume.

To test the biological activity of p28, we made use of the cytokine-dependent growth of murine pre-B cell line Ba/F3 stably transfected with cDNAs coding for gp130 (resulting in Ba/F3-gp130) and either human or murine IL-6R (resulting in Ba/F3-gp130-hIL-6R or Ba/F3-gp130-mIL-6R, respectively). STAT1 and STAT3 phosphorylation as well as cytokine-dependent proliferation of Ba/F3-gp130 cells were induced by Hyper-IL-6, a fusion protein of IL-6 and the soluble IL-6R, and in Ba/F3-gp130-IL-6R cells by Hyper-IL-6 or IL-6 (Fig. 1, C and D) (11). Murine IL-6 (10 ng/ml) induced only proliferation in Ba/F3-gp130-mIL-6R cells, whereas human IL-6 (10 ng/ml) induced STAT1/STAT3 phosphorylation and proliferation in Ba/F3-gp130-hIL-6R and Ba/F3-gp130-mIL-6R cells (10, 15). Bacterially expressed p28 combined with EBI3 formed the active IL-27 cytokine, as shown by p28/EBI3-induced proliferation of Ba/F3-gp130 cells (supplemental Fig. 1F). p28 alone, however, also induced STAT1/3 phosphorylation and dose-dependent proliferation of both Ba/F3-gp130-mIL-6R and Ba/F3-gp130-hIL-6R cells (Fig. 1, C–E). Dose-dependent p28-induced proliferation of Ba/F3-gp130-mIL-6R and Ba/F3-gp130-hIL-6R cells (0–4000 ng/ml p28) showed that a 3.5-fold lower concentration of p28 was needed to induce half maximal proliferation of Ba/F3-gp130-mIL-6R cells (4 nM) compared with half maximal proliferation of Ba/F3-gp130-hIL-6R cells (13.7 nM). This result indicates that the affinity of murine p28 to murine IL-6R is higher compared with human IL-6R (Fig. 1E). Ba/F3-gp130 cells lacking the IL-6R did not show STAT1/STAT3-phosphorylation or proliferation when stimulated with 200 ng/ml p28 alone (Fig. 1, C and D). These results showed that bacterially expressed murine p28 was biologically active and, in contrast to murine IL-6, induced proliferation via binding to the murine and human IL-6R.

The anti-hIL-6R monoclonal antibody tocilizumab, which is approved for treatment of rheumatoid arthritis and other dis-

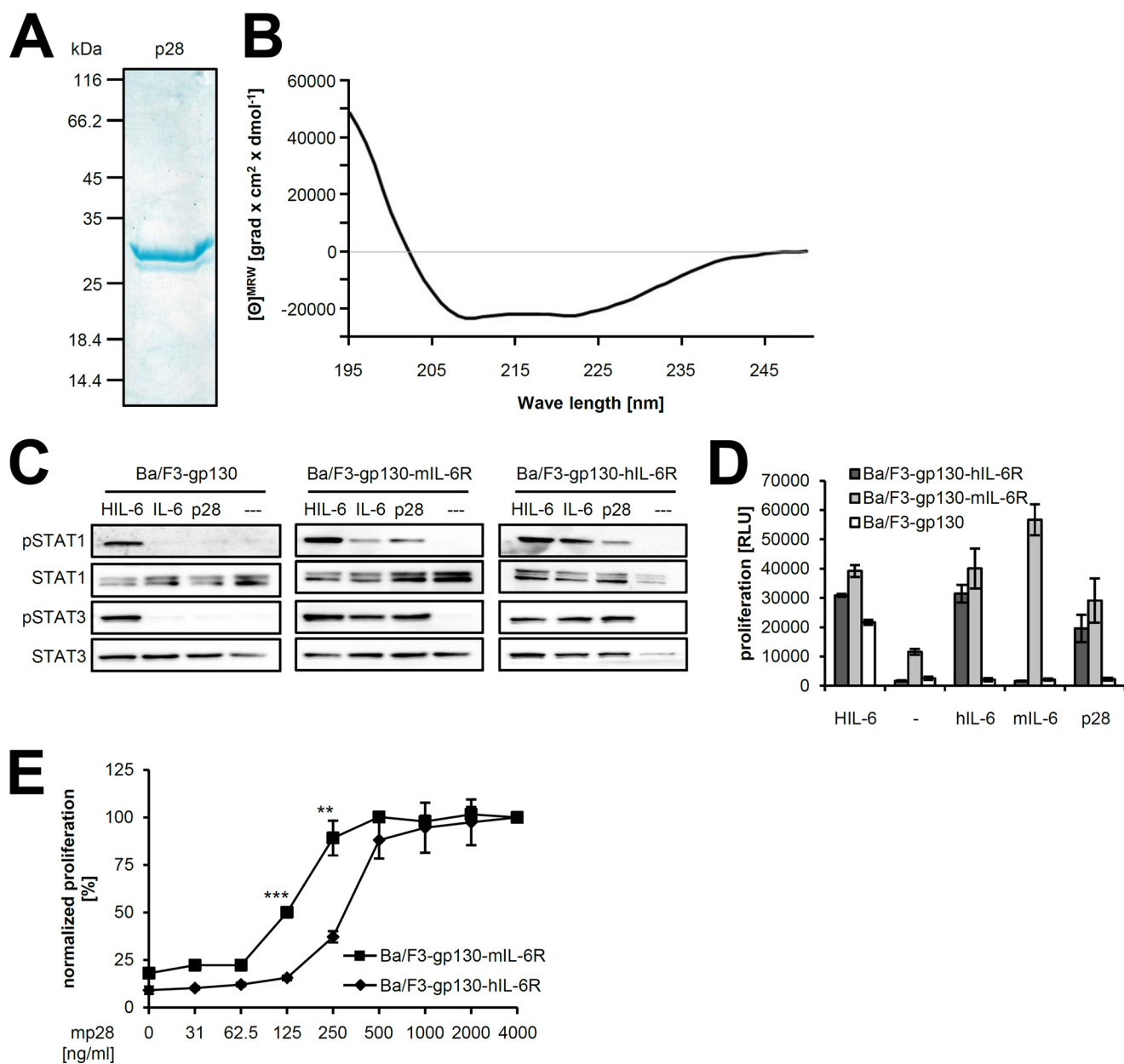


FIGURE 1. Bacterially produced p28 is an agonistic cytokine. *A*, SDS-PAGE and Coomassie staining of purified monomeric p28. Two bands containing p28 of slightly differing size were visible. The weaker and lower band contains most probably degraded p28. *B*, CD spectrum of refolded p28. The shape of the spectrum indicated an α -helical conformation. *C*, equal numbers of Ba/F3-gp130, Ba/F3-gp130-hIL-6R, and Ba/F3-gp130-mIL-6R cells were stimulated for 15 min with 10 ng/ml Hyper-IL-6 (HIL-6), 10 ng/ml human IL-6, or 200 ng/ml murine p28 or were left unstimulated. Phosphorylation of STAT1 and STAT3 was assessed by Western blotting. Determination of STAT1 and STAT3 protein levels served as loading controls. *D*, equal numbers of Ba/F3-gp130, Ba/F3-gp130-hIL-6R, and Ba/F3-gp130-mIL-6R cells were cultured for 2 days with 10 ng/ml Hyper-IL-6, 10 ng/ml human IL-6 (hIL-6), 10 ng/ml murine IL-6 (mIL-6) or 200 ng/ml murine p28 (p28) or were left unstimulated. *E*, equal numbers of Ba/F3-gp130-hIL-6R and Ba/F3-gp130-mIL-6R cells were cultured for 2 days with different concentrations of murine p28 (0–4,000 ng/ml). Cellular proliferation in *D* and *E* was quantified as indicated under "Experimental Procedures."

eases, is directed against the cytokine-binding module (CBM) of the human IL-6R and prevents binding of IL-6 to the IL-6R via site I of IL-6 and inhibited IL-6-induced proliferation of Ba/F3-gp130-hIL-6R cells (10). We stimulated Ba/F3-gp130-hIL-6R cells with 200 ng/ml p28 or 10 ng/ml IL-6 in the presence of increasing concentrations of tocilizumab (0–1000 ng/ml). Tocilizumab blocked p28- and IL-6-induced proliferation of Ba/F3-gp130-hIL-6R cells in a similar dose-dependent manner (Fig. 2A), indicating that IL-6 and p28 use the same IL-6R-binding interface. Because p28 can be secreted in complex with cytokine-like factor (CLF) 1 or EBI3, local or circulat-

ing free p28/CLF complexes might induce signaling on IL-6R expressing cells *in vivo* as part of the inflammatory reaction chain, which, as a consequence, would also be inhibited by therapeutic application of tocilizumab. Next, we tested the antagonistic properties of p28. Proliferation of Ba/F3-gp130 cells, which was induced by Hyper-IL-6 or IL-27, was not suppressed, even by an about 500-fold molar excess of p28 (Fig. 2, B and C). Moreover, proliferation of Ba/F3-gp130-mIL-6R cells, induced by human or murine IL-6, was also not suppressed by an about 40-fold molar excess of p28 (Fig. 2D). Taken together, our results indicate that bacterially produced murine p28 acts as an

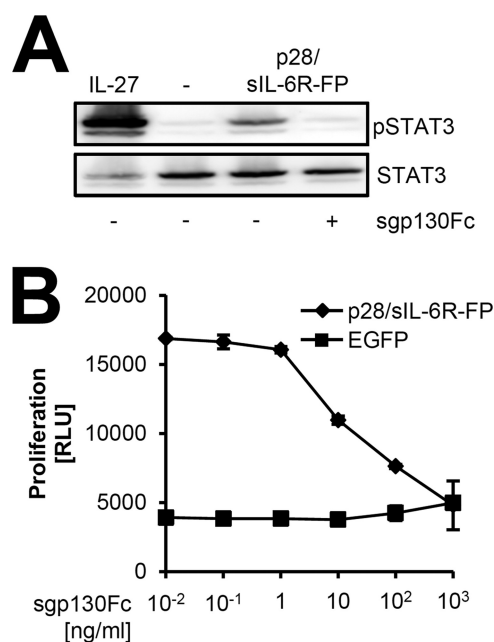


FIGURE 3. p28 trans-signaling via the soluble IL-6R is inhibited by sgp130Fc. *A*, equal numbers of Ba/F3-gp130 cells were stimulated with p28/siL-6R-FP- or IL-27-conditioned media from transiently transfected COS7 cells for 15 min or left untreated. Conditioned medium was supplemented with sgp130Fc 30 min prior to stimulation where indicated. Cell lysates were analyzed via Western blotting with antibodies against pSTAT3 and against STAT3 as a loading control. *B*, equal numbers of Ba/F3-gp130 cells were cultured for 2 days with p28/siL-6R-FP-conditioned medium in the presence or absence of increasing concentrations of sgp130Fc (0.01–1000 ng/ml). As a control, supernatant from enhanced GFP-expressing (EGFP) COS7 cells was used. Cellular proliferation was quantified as indicated under “Experimental Procedures.”

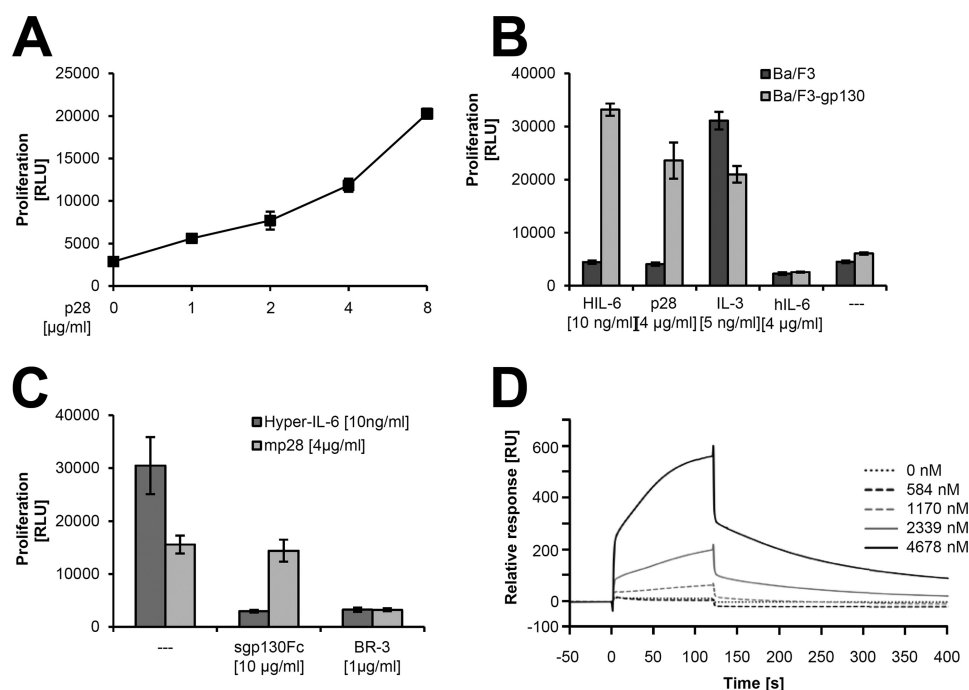


FIGURE 4. p28 can signal via gp130 without the α -receptors EB13 and IL-6R. *A*, equal numbers of Ba/F3-gp130 cells were incubated with increasing concentrations of p28 (0–8000 ng/ml) for 2 days. *B*, equal numbers of Ba/F3 or Ba/F3-gp130 cells were cultured for 2 days with cytokines at the indicated concentrations or left unstimulated. *hIL-6*, human IL-6; *HIL-6*, Hyper-IL-6. *C*, equal numbers of Ba/F3-gp130 cells were cultured for 2 days with either 10 ng/ml Hyper-IL-6 or 4 μ g/ml p28. sgp130Fc (10 μ g/ml) and BR-3 (1 μ g/ml) were tested for their ability to inhibit p28-induced Ba/F3-gp130 proliferation. In *A–C*, cellular proliferation was quantified as indicated under “Experimental Procedures.” *D*, surface plasmon resonance measurements of binding of p28 to sgp130Fc as indicated under “Experimental Procedures.”

responsive cells to virtually all cells of the body in analogy to IL-6. Furthermore, sgp130Fc has been shown to be effective for the treatment of chronic inflammatory diseases in mice and is currently under development for therapeutic application in patients (1). However, it has to be taken into consideration that the IL-6 trans-signaling inhibitor sgp130Fc is also an inhibitor of p28/siL-6R trans-signaling, just as the complete IL-6 inhibitor tocilizumab also completely inhibits p28 signaling via IL-6R.

p28 Is a Biologically Active Cytokine Even in the Absence of the α -Receptors EB13 and IL-6R—Next, we asked if p28 can induce signaling in gp130- and Wsx-1-expressing cells without the need of an α -receptor. We showed that low concentrations of p28 (200 ng/ml) induced proliferation of Ba/F3-gp130-IL-6R cells but did not induce proliferation of Ba/F3-gp130 cells (Fig. 2*B*). Surprisingly, concentrations starting from 1 μ g/ml of p28 induced proliferation of Ba/F3-gp130 cells in a dose-dependent manner (Fig. 4*A*). p28-mediated proliferation was dependent on the β -receptor chain gp130 because Ba/F3 cells lacking gp130 expression did not proliferate in the presence of p28 (Fig. 4*B*). Parallel experimental controls showed that a high concentration of IL-6 (4 μ g/ml), also produced in *E. coli*, did not induce proliferation of Ba/F3 or Ba/F3-gp130 cells (Fig. 4*B*). In contrast, both cell types proliferated in response to their natural growth factor IL-3 (Fig. 4*B*). Hyper-IL-6 selectively stimulated proliferation in Ba/F3-gp130 cells (Fig. 4*B*) (11). The involvement of gp130 in p28-mediated signaling was proven by inhibition of p28-induced proliferation of Ba/F3-gp130 cells with the neutralizing anti-gp130 mAb BR-3 (Fig. 4*C*). Interestingly, we were not able to inhibit p28-mediated proliferation of

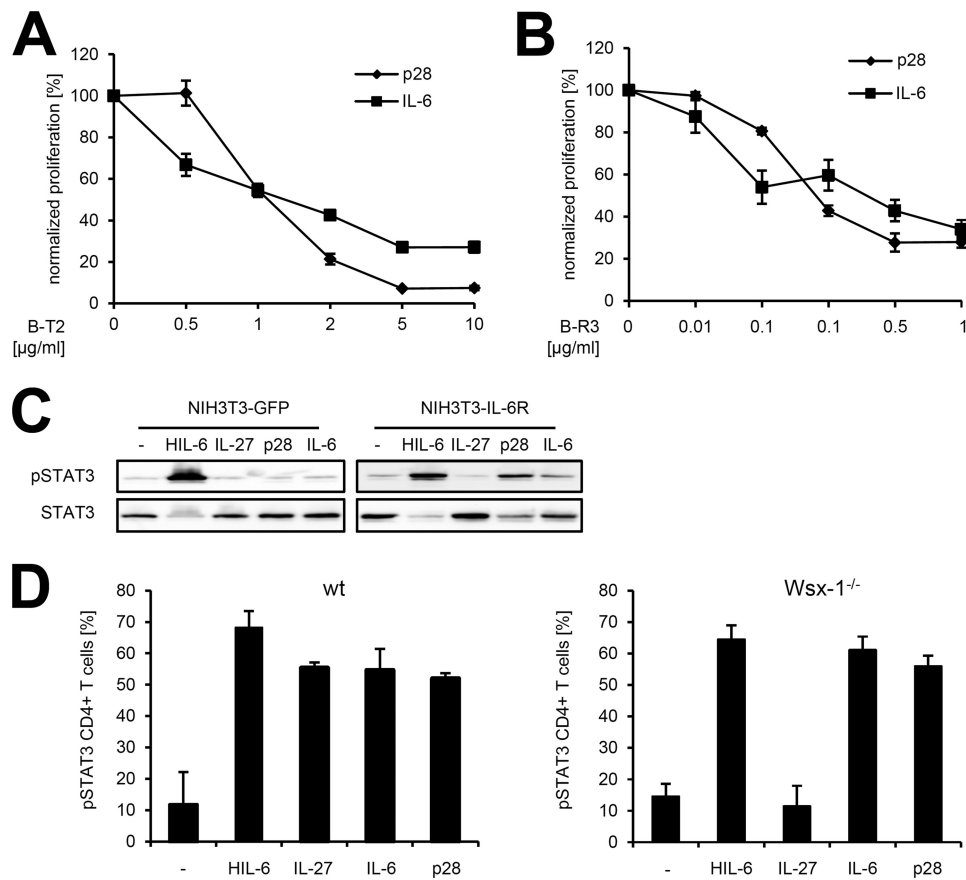


FIGURE 5. p28/IL-6R-induced signaling is mediated by a homodimer of the β -receptor chain gp130. *A*, Ba/F3-gp130-mIL-6R cells were cultured for 2 days with 10 ng/ml human IL-6 (*hIL-6*) or 200 ng/ml murine p28 and increasing concentrations of the gp130 antibody BT-2 (0–10 μ g/ml). *B*, Ba/F3-gp130-mIL-6R cells were cultured for 2 days with 10 ng/ml human IL-6 or 200 ng/ml murine p28 and increasing concentrations of the gp130 antibody BR-3 (0–1 μ g/ml). In *A* and *B*, cellular proliferation was quantified as indicated under “Experimental Procedures.” *C*, NIH3T3 cells were transiently transfected with plasmids coding for the human IL-6R or enhanced GFP (*EGFP*). Cells were stimulated with Hyper-IL-6 (*HIL-6*, 10 ng/ml), IL-27 (10 ng/ml), p28 (200 ng/ml), or IL-6 (50 ng/ml) for 15 min or were left untreated. Phosphorylation of STAT3 was assessed by Western blotting. Determination of STAT3 protein levels served as loading control. *D*, purified CD4⁺ T cells from wild-type and from *Wsx-1*-deficient mice were stimulated with Hyper-IL-6 (10 ng/ml), IL-27 (10 ng/ml), IL-6 (10 ng/ml), or p28 (200 ng/ml) for 15 min or were left untreated. STAT3 phosphorylation was determined by flow cytometry and normalized.

Ba/F3-gp130 cells with *sgp130Fc* (Fig. 4C). By using surface plasmon resonance, we could detect binding of p28 to *sgp130Fc* with a K_D of 190 μ M (Fig. 4D). In comparison, the affinity of Hyper-IL6 to *sgp130Fc* had been determined to be 4.99×10^{-10} M (14). Even though the affinity of p28 to gp130 is low, our data indicate that p28 can induce signaling via binding to gp130 even in the absence of IL-6R or EBI3. Furthermore, the low affinity of p28 to gp130 makes it highly unlikely that p28 is able to act as an inhibitor of IL-6 by competing for gp130 with IL-6.

*p28/IL-6R-induced Signaling Is Mediated by the β -receptor Chain gp130 but Not by *Wsx-1**—Signaling of p28 via EBI3 (IL-27) is mediated by a heterodimer of gp130 and *Wsx-1*, whereas signaling of IL-6 via the IL-6R is mediated by a homodimer of gp130. IL-6 binds to the Ig-like domain of one gp130 receptor molecule and to the CBM of the second gp130 receptor molecule. Because *Wsx-1* contains only the CBM but no Ig-like domain, IL-27 signaling must be mediated by binding to the Ig-like domain of gp130 and to the CBM of *Wsx-1*. To characterize the p28/IL-6 β -receptor complex, Ba/F3-gp130-IL-6R cells were incubated with p28, IL-6, and IL-27 in the presence or absence of the neutralizing anti-gp130 mAbs BT-2 and BR-3. BT-2 is directed against the Ig-like domain, and BR-3 is directed against the CBM (domains 2 and 3) of gp130 (21, 22). To verify

the ability of the two antibodies to discriminate between signaling via gp130/gp130 and gp130/*Wsx-1*, we incubated Ba/F3-gp130 cells with either Hyper-IL-6 or IL-27 and BT-2 or BR-3 (Supplemental Fig. 3, A and B). As expected, the BT-2 antibody was able to inhibit proliferation of Ba/F3-gp130 cells incubated with either Hyper-IL-6 or IL-27 because the Ig-like domain of gp130 is needed to initiate a functional signaling complex in both cases. In contrast, BR-3 only inhibited Hyper-IL-6-mediated proliferation, whereas IL-27-induced proliferation was not affected because the CBM of gp130 is not involved in IL-27 signaling. Interestingly, p28-as well as IL-6-induced signaling was inhibited in a dose-dependent manner by BT-2 and BR-3 (Fig. 5, A and B). It was shown previously that p28 plus EBI3 (IL-27) needed the Ig-like domain of gp130 for signal transduction (5). If p28/IL-6R induced a heterodimer of *Wsx-1* and gp130, p28 would have to bind to the CBM of gp130 and to the CBM of *Wsx-1*. However, no cytokine of the IL-6 family has been shown to simultaneously bind to two CBMs. Therefore, the BR-3-inhibition experiment raised the possibility that *Wsx-1* might not be involved in signaling of p28 via the IL-6R. To further investigate the involvement of *Wsx-1* in p28/IL-6R receptor complex formation, we analyzed p28-induced signaling in cells lacking *Wsx-1* expression. We found that NIH3T3

cells express gp130 but lack Wsx-1 expression (supplemental Fig. 4, A–F). Because of the lack of Wsx-1 and IL-6R expression, NIH3T3 cells were responsive to Hyper IL-6 but not to IL-27, IL-6, and p28 as analyzed by STAT3 phosphorylation (Fig. 5C). After transient transfection of an expression plasmid for human IL-6R, these cells became responsive to IL-6 and p28, but still did not react to IL-27 (Fig. 5C). Next, CD4⁺ T-cells from wild-type and Wsx-1-deficient mice were stimulated with p28, IL-27, IL-6, or Hyper-IL-6, and STAT3 phosphorylation was quantified by flow cytometry. As shown in Fig. 5D, p28-, IL-6- and Hyper-IL-6-induced STAT3 phosphorylation in T cells from wild-type and Wsx-1-deficient mice, whereas IL-27 only induced STAT3 phosphorylation in T cells from wild-type mice. Taken together, these results showed that Wsx-1 is not involved in p28/IL-6R-mediated receptor complex formation but that p28 rather uses a gp130 homodimer for signal transduction.

DISCUSSION

In this study, several potentially important findings are described for the first time. We developed a robust protocol for the production of the biologically active and correctly folded IL-27 cytokine subunit p28 in *E. coli*. This is important for research in the field because p28 alone is poorly secreted from human cells (24), which so far impaired the analysis of p28-mediated effects *in vitro* and *in vivo*. Previously, biologically active p28 was produced by a cosecretion system with CLF in HEK293 or insect cells. It was postulated that binding of p28 to IL-6R is mediated via site I of p28 and that CLF likely bound to site III of p28 is replaced by one of the signaling β -receptors (7). In conclusion, it was assumed that CLF was only needed for secretion of p28 but not for p28/IL-6R-mediated signaling (7). Even though bacterially produced p28 was used in two previous studies, the authors did not describe the production or the refolding procedure of p28 and did not present any data on its folding and oligomerization status (7, 8). In addition, experiments showing that the recombinant p28 was able to form biologically active IL-27 together with EBI3 were missing in both studies. Our p28 was monomeric, correctly folded as judged by circular dichroism spectroscopy, and biologically active. In line with Crabé *et al.* (7), but in contrast to Stumhofer *et al.* (8), bacterially produced p28 acts as an agonist by inducing signal transduction dependent on membrane-bound murine and human IL-6R. Inhibitor studies with tocilizumab indicated that the affinity of p28 to the IL-6R is much lower than the affinity of IL-6 to IL-6R.

Another important novel finding of this study is that p28 is able to form a biologically active composite cytokine with sIL-6R. The functional role of the sIL-6R *in vivo* is so far restricted to IL-6 trans-signaling. IL-6 trans-signaling enables IL-6 to activate cells that lack the IL-6R and that would normally not respond to this cytokine. Because gp130 is ubiquitously expressed, the IL-6/sIL-6R complex can also stimulate cells that are nonresponsive to IL-6 alone (2). IL-6 remains the only example of a cytokine that *in vivo* uses both classic and trans-signaling via the soluble IL-6R, even though IL-11 and ciliary neurotrophic factor have been shown to be able to mediate signals via the sIL-11R and soluble ciliary neurotrophic factor receptor *in vitro*, respectively (25, 26). The natural sgp130 pro-

tein and sgp130 designer proteins like sgp130Fc specifically block IL-6 trans-signaling without sterically affecting classic signaling because IL-6 alone has no measurable affinity to gp130. IL-6 trans-signaling drives inflammatory events, and the beneficial effect of sgp130 and sgp130Fc has been documented in experimental models of arthritis, colitis, infection, allergy, and inflammation-induced cancer (1). Under pathophysiological conditions, sIL-6R levels can rise from 10–50 ng/ml to 100–200 ng/ml. Moreover, the p28 serum level of 100 ng/ml and more were described in mice after infection with *Toxoplasma gondii* (8), theoretically enabling p28 trans-signaling *in vivo*. Here we show that p28 trans-signaling was inhibited by sgp130Fc in a dose-dependent manner. Apart from IL-6, no other IL-6 type cytokine, including IL-27, is inhibited by sgp130(Fc). The selective difference of sgp130 for p28/sIL-6R and IL-27 suggested a different receptor usage of these two cytokines. Accordingly, we found that p28 has an intrinsic affinity to gp130 and that, albeit at higher concentrations, p28 is able to activate signal transduction via gp130 in the absence of IL-6R or EBI3. Future studies will, however, have to elucidate the biological significance of p28/IL-6R classic signaling, p28/sIL-6R trans-signaling, and p28 signaling *in vivo*.

The heterodimeric IL-6-type cytokine IL-27 induces the dimerization and activation of the β -receptor complex composed of gp130 and Wsx-1, whereas IL-6 signaling via the IL-6R is mediated by a gp130 homodimer. The receptor composition of p28/IL-6R was until now not identified (7). The proposed gp130/Wsx-1 heterodimer seemed to be likely, but was recently challenged (27). Dibra *et al.* (27) demonstrated that the beneficial effects of p28 in the treatment of liver injury were also evident in Wsx-1-deficient mice, making the signaling of p28 via a gp130/Wsx-1 heterodimer unlikely. Using antibodies and gene-deficient cell lines, we identified the p28 receptor complex to be composed of IL-6R and a gp130 homodimer. Wsx-1 was not involved in the signal transduction of p28/IL-6R. This might indicate that p28 acts more like the cytokines IL-6 and IL-11, also signaling via a gp130 homodimer, rather than like IL-27.

Like OSM, which can signal via the two different β -receptor complexes OSM receptor-gp130 and leukemia inhibitory factor receptor-gp130 (1), p28 is the second example of a β -receptor binding interface plasticity, allowing the binding of p28 site II to the CBM of Wsx-1 or to the CBM of gp130. In contrast to OSM, the binding of p28 to gp130 or Wsx-1 is not stochastic but highly selective and controlled by presence of the α -receptors EBI3 and IL-6R. How this selectivity is regulated on the molecular level is, however, not understood. It was recently shown for IL-6 and its homologue viral IL-6 (encoded by the human herpesvirus 8) that an α -receptor can influence the conformation of a cytokine binding site (28). Viral IL-6 can bind to a gp130 homodimer even in the absence of IL-6R. Transferring of the complete binding site III from viral IL-6 to IL-6 resulted in an IL-6/viral IL-6 chimeric protein that also bound to gp130 without the need of the IL-6R (23). These results suggested that the binding of the IL-6R to IL-6 somehow modifies site III (and in p28 maybe also site II) into a gp130-binding conformation. This mechanism might have evolved to prevent overwhelming cellular activation by IL-6. Binding of a cytokine such as p28 to

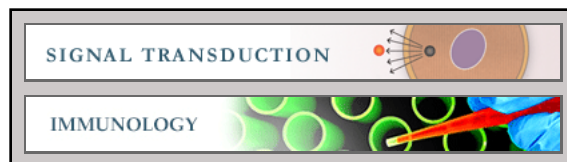
two different β -receptor combinations in dependence of the α -receptor is, however, without example so far. Future studies should include the comparison of the solution structures of p28/EBI3 and p28/sIL-6R, which might shed light on the structural differences between the p28 β -receptor binding sites.

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Signal Transduction:
**An Interleukin-6 Receptor-dependent
Molecular Switch Mediates Signal
Transduction of the IL-27 Cytokine
Subunit p28 (IL-30) via a gp130 Protein
Receptor Homodimer**



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J. Biol. Chem. 2013, 288:4346-4354.

doi: 10.1074/jbc.M112.432955 originally published online December 3, 2012

Access the most updated version of this article at doi: [10.1074/jbc.M112.432955](https://doi.org/10.1074/jbc.M112.432955)

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