SHP2 and SOCS3 Contribute to Tyr-759-dependent Attenuation of Interleukin-6 Signaling through gp130*

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Interleukin-6 (IL-6) activates the Jak/STAT pathway as well as the mitogen-activated protein kinase cascade. Tyrosine 759 of the IL-6 signal-transducing receptor subunit gp130 has been identified as being involved in negative regulation of IL-6-induced gene induction and activation of the Jak/STAT pathway. Because this site is known to be a recruitment motif for the protein-tyrosine phosphatase SHP2, it has been suggested that SHP2 is the mediator of tyrosine 759-dependent signal attenuation. We recently observed that the suppressor of cytokine-signaling SOCS3 also acts through the tyrosine motif 759 of gp130. However, the relative contributions of SHP2 and SOCS3 to the repression of IL-6 signaling are not understood. Therefore, we designed experiments allowing the independent recruitment of each of these proteins to the IL-6-receptor complex. We show that receptor- and membrane-targeted SHP2 counteracts IL-6 signaling independent of SOCS3 binding to gp130. On the other hand, SOCS3 inhibits signaling in cells expressing a truncated SHP2 protein, which is not recruted to gp130. These data suggest that there are two, largely distinct modes of negative regulation of gp130 activity, despite the fact that both SOCS3 and SHP2 are recruited to the same site within gp130.

Interleukin-6 (IL-6) is a cytokine with a wide spectrum of activities. It stimulates the differentiation of B- and T-cells and the proliferation of keratinocytes, mesangial and plasmacytoma cells, whereas the proliferation of breast carcinoma cells, as well as melanoma cells is inhibited (for review see Ref. 1). IL-6 is the major regulator of the expression of acute-phase protein genes in liver cells (2–5) and exerts its action by binding and activating a receptor complex composed of a specific α-subunit (IL-6Rα, gp80, or CD126) and the signal transducing subunit gp130 (CD130) (6, 7). Ligand binding to the α-subunit leads to the dimerization of gp130 and the activation of the constitutively associated Janus kinases Jak1, Jak2, and Tyk2 (8, 9). In turn, gp130 becomes tyrosine-phosphorylated on its cytoplasmic tail and recruits transcription factors of the family of signal transducers and activators of transcription (STAT), STAT1 and STAT3, to specific phosphotyrosine motifs (10, 11). Subsequently, STAT factors become tyrosine-phosphorylated, dissociate from the receptor complexes, and translocate to the nucleus, where STAT homo- and/or heterodimers bind to specific DNA elements in the promoters of IL-6 target genes (12, 13).

Tyrosine 759 of gp130 has been suggested to be involved in the inhibition of IL-6 signaling: mutation of tyrosine 759 to phenylalanine was shown to enhance signal transduction of IL-6 (14–16) as well as signaling of leukemia inhibitory factor and oncostatin M (17). The latter cytokines signal through LIF-R/gp130 or OSM-R/gp130 heterodimeric receptor complexes, respectively. Mice expressing gp130, which lack the tyrosine 759, display splenomegaly, lymphadenopathy, and an enhanced acute phase reaction (18). On the other hand, mice expressing C-terminally deleted gp130 lacking the STAT-binding sites show a similar phenotype as IL-6-deficient mice (impaired humoral and mucosal immune and hepatic acute phase response) (19).

The protein-tyrosine phosphatase SHP2 is believed to mediate Tyr-759-dependent signal attenuation, because SHP2 is recruited to phosphotyrosine 759 after receptor activation and becomes tyrosine-phosphorylated (14–16). Mutation of Tyr-759 impairs SHP2 recruitment and phosphorylation (10). Furthermore, IL-6 induced activation of the MAPK cascade is blocked by this receptor mutation. SHP2 is a ubiquitously expressed cytoplasmic protein-tyrosine phosphatase containing two N-terminal Src homology 2 (SH2) domains and a phosphatase domain in the C-terminal half. The crystal structure of SHP2 suggests that, in the absence of a tyrosine-phosphorylated binding partner, the N-SH2 domain blocks the catalytic domain (20). Recently, Lu et al. (21) presented a mechanism for the
regulation of SHP2 activity, suggesting that the N-SH2 domain interacts with the PTP domain to inhibit phosphatase activity. SHP2 becomes activated by the phosphorylation of tyrosine 542 or 580. Subsequently, these phosphotyrosines interact with the N- and C-terminal SH2 domain, respectively, relieving the PTP domain from the N-SH2 domain-mediated inhibition (21). Binding of the SH2 domains to phosphopeptides deduced from receptors or adapter molecules induces enzymatic activity (22–24).

SOCS3 belongs to the family of suppressors of cytokine signaling (SOCS) proteins. The members of this protein family (CIS and SOCS1 through SOCS7) contain an N-terminal SH2 domain, preceded by the extended SH2 domain (ESS) and the kinase inhibitory region (KIR). Furthermore, a homology domain called SOCS-box is located at the C terminus (25–30). Primarily, SOCS1 and SOCS3 genes are rapidly induced by IL-6 and are potent inhibitors of IL-6-mediated signaling. Thus, they are regarded as classic feedback inhibitors (25–27). Several mechanisms by which SOCS proteins inhibit cytokine signaling have been proposed: SOCS1, and to some extent SOCS3, have been found to inhibit the kinase activity of Jak5, probably by binding to the activation loop of the kinase (30, 31). Another model for SOCS function proposes the targeting of signaling components to proteasome-dependent degradation (32, 33). The latter is in line with the short half-lives of SOCS proteins (34).

The contribution of SHP2 to tyrosine 759-dependent signal attenuation has to be re-examined because we found that SOCS3 also acts through and binds to the phosphotyrosine motif 759 of gp130 (35). Actually, the affinity of SOCS3 to a phosphotyrosine peptide corresponding to the Tyr-759 motif of gp130 is much higher than to a phospho-peptide comprising the activation loop of the Janus kinase (36). Furthermore, the affinity of SOCS3 to bind gp130 is even slightly higher than that of SHP2 (37). Although SOCS3-SHP2 complexes have been observed, the interaction of SOCS3 and gp130 appears to be SHP2-independent (38).

It was the goal of this study to dissect the contributions of SHP2 and SOCS3 to Tyr-759-dependent signal attenuation. We asked whether SHP2 is involved in the Tyr-759-mediated repression of IL-6 signaling and whether SHP2 and SOCS3 act independently from each other.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and Hybaid (Heidelberg, Germany). Oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Vent polymerase and antibodies specific for activated STAT3 were obtained from New England BioLabs (Beverly, MA). Recombinant erythropoietin (Epo) was a generous gift of Drs. J. Burg and M. S. Mak (Cambridge, MA). Oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany) and MWG-Biotech (Ebersberg, Germany). Recombinant IL-6 and sIL-6R were prepared as described previously (38). The specific activity of IL-6 was 2 × 10^{9} B-cell-stimulatory factor (2 μg/mg of protein).

**Construction of Expression Vectors**—Cloning was carried out by standard procedures. pG3A-M-215Luc contains the promoter region −215 to +8 of the rat α−macroglobulin fused to the luciferase encoding sequence and was described previously (15). The SIE-th-Luc construct, containing two copies of the STAT consensus binding sequence from the c-fos promoter upstream of a thymidine kinase minimal promoter (48), was kindly provided by Hugues Gascan (Angers, France). Expression vectors for the IL-5Rα/gp130 receptor chimeras pRCMV-IL-5Rα/gp130(YYYYY), pRCMV-IL-5Rα/gp130(FYYYYY), pRCMV-IL-5Rα/gp130(FYYYYY), and pRCMV-IL-5Rα/gp130(YFYYYY) were used in transient transfection experiments as described previously (17). pRCMV-IL-5Rα/gp130(YFYYYY) lacks amino acids 766–918 of gp130. For construction of the pRCMV-IL-5Rα/gp130(YFYYYY) vector, the cDNA of an SHP2 protein lacking the N-terminal 208 amino acids was amplified by PCR. This fragment was fused to the 3′-end of the cDNA of the IL-5Rα/gp130(YF) vector by simultaneous elimination of the stop codon. For steric flexibility a linker sequence of glycine and serine residues (GS)G, was introduced between the residual part of gp130 and the SHP2 fragment giving pRCMV-IL-5Rα/gp130(YF-PTP). The construction of pBC1-SHP2WT and pBC1-SHP2C→S was carried out as previously described (15). pBC1-SHP2D→A was generated by a gene exchange fragment containing an SnaBI site located at least in triplec. Luciferase reporter vector pBC1-V-PTP encodes a mutant of human SHP2 lacking both SH2 domains (amino acids 1–208). For membrane targeting the myristoylation signal MGCMMKSKFLQ of the murine hematopoietic cell kinase (hck) proto-oncogene p59 was fused to the N terminus. The expression vector pBC1-V-PTP is identical to pBC1-V-PTP but lacks the myristoylation signal. These constructs were cotransfected for reporter gene assays with previously described EpoR/gp130 receptor chimeras (pRCMV-EG(YYYYY) and pRCMV-EG(YYYYYY)) (15). Jak1 expression vector (pSVL-Jak1) was described previously (40). The expression vector for murine SOCS3 was pEF-FLAG-1mSOCS3 (26), kindly provided by D. Hilt, Melbourne, Australia. The SOCS3 mutants (SOCS3box, S3ES, SOCS3-36, and S3ES4) were generated by exchange of the full-length SOCS3-cDNA by of DNA fragments coding for amino acids 1–183, 23–225, or 44–225 of SOCS3, respectively. The SOCS3 deletion mutants S3A20, S3A36, S3A40, S3C84, and point mutations S3L22D, S3F25A, S3L41R, S3G45A, S3R71K, and S3R71E were described previously (41) (S3 stands for SOCS3).

**Transfection and Reporter Gene Analysis**—Human HepG2 hepatoma cells were grown and transiently transfected by the calcium phosphate coprecipitation method as described previously (42). Transfections were adjusted with control vectors to equal amounts of DNA. Cell lysis and luciferase assays were carried out using the luciferase kit (Promega, Madison, WI) as described by the manufacturer. All transient expression experiments were done at least in triplicate. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector (pCR3lacZ, Amersham Biosciences, Uppsala, Sweden) (1.5 μg). COS-7 cells were grown in DMEM with 10% FCS, 100 μg/ml streptomycin, and 60 μg/ml penicillin. Approximately 1.5 × 10^{5} COS-7 cells were transiently transfected with 6–25 μg of DNA using the DEAE-dextran method. Briefly, cells were incubated in medium containing the DNA, 80 μg chloroquine, and 0.4 mg/ml DEAE-dextran for 80 min avoiding gas exchange. Afterward, cells were incubated for 1 min in phosphate-buffered saline containing 10% MeSO. After 24 hr cells were split 1:2, and, after additional 24 h in culture medium, cells were stimulated. 3T3 embryonic fibroblasts and wild-type mice were used as controls. 3T3 fibroblasts and wild-type mice were transfected with luciferase reporter vectors containing a Gal4-promoter element was cotransfected with a cotransfected β-galactosidase expression vector (pCR3lacZ, Amersham Biosciences) as described (15). COS-7 cells were grown in DMEM with 10% FCS, 100 μg/ml streptomycin, and 60 μg/ml penicillin (43). These cells were transiently transfected with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) as described in the manufacturer’s instructions. MAPK activity was monitored by phosphorylation of Elk1 transcription factor (pFA2-Elk1). Transfection efficiency was monitored by using a cotransfected β-galactosidase expression vector as described above. Activated MAPKs lead to phosphorylation of the Elk transactivation domain and thus to expression of luciferase.
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Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—The preparation of nuclear extracts, measurements of protein concentrations, and EMSAs have been described previously (44). For STAT1- and STAT3-specific double-stranded \(^{32}\)P-labeled probes, we used a mutated SIE oligonucleotide of the c-fos promoter (m67 SIE, 5′-GATCCGGGAGGGTTAAGGAGAAGG-3′) (45). Protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25× TBE at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 30 min, dried, and autoradiographed.

Immunoprecipitation and Immunoblot Analysis—For immunoprecipitations 2 × 10\(^5\) cells were lysed in 500 μl of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl; 1% Triton X-100; 10% glycerol; 20 mM Tris/HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA). Buffers were supplemented with aprotinin, pepstatin, and leupeptin (10 μg/ml of each). Equal amounts of cellular protein were incubated with the appropriate antibodies or with 2 μM biotinylated peptides at 4 °C overnight and precipitated with 2.5 mg of Protein A-Sepharose (Amersham Biosciences, Sweden) or NeutrAvidin-coupled agarose (Pierce, Rockford, IL), respectively. Immune complexes were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Antigens were detected by incubation with the appropriate primary antibodies (PY99, 1:100; 4G10, 1:1000; anti-SHP2, 1:100; anti-Jak1, 1:1000; anti-FLAG2-m2-biotin, 1:500; anti-Myc, 1:1000; IL-5Rα, 1:1000; and IL-5Rγ, 1:1000) and horseradish peroxidase-coupled secondary antibodies (1:2000) (Dako, Hamburg, Germany) or horseradish peroxidase-coupled streptavidin (1:5000) (Pierce, Rockford, IL). The membranes were developed with an enhanced chemiluminescence kit (Amersham Biosciences, Freiburg, Germany). Blots were stripped and reprobed to verify application of equal amounts of protein.

Assay of SOCS3 in Bacteria—Human SOCS3 was expressed as a thioredoxin fusion protein in BL21(DE3) Escherichia coli (Stratagene, Heidelberg, Germany). Bacteria were grown in LB medium containing 100 μg/ml ampicillin at 37 °C to an A\(_{600}\) of 1 and then induced with 1 mM isopropyl-1-thio-β-galactopyranoside. Cells were harvested after 3 h of expression, resuspended in 50 mM Tris/HCl, pH 9.0, 10% glycerol, and processed for SDS-PAGE. SOCS3 was purified on a HiTrap chelating 5-ml column (Amersham Biosciences, Freiburg, Germany) with nickel/imidodiacetic acid (NI-IDA) as matrix. Native eluted SOCS3 was dialyzed into 50 mM Tris, 10 mM dithiothreitol, pH 8.5 and purified to homogeneity by anion-exchange chromatography on a Mono Q column (Amersham Biosciences, Freiburg, Germany). For biosensor measurements the protein was dialyzed against 50 mM Tris/HCl, pH 8.0, 10 mM dithiothreitol, 0.05% Chaps. Purity of the recombinant protein was monitored by SDS-PAGE.

Biosensor Analysis—Biotinylated peptides were loaded on a strept-Avidin-coated Biosensor chip (Biacore, Freiburg, Germany). The amount of loaded peptide was 80 ± 4 fmol/mm\(^2\) chip surface, which corresponds to 141–159 pmol peptide/ml. SOCS3 was purified on a HiTrap chelating 5-ml column (Amersham Biosciences, Freiburg, Germany) with nickel/imidodiacetic acid (NI-IDA) as matrix. Native eluted SOCS3 was dialyzed into 50 mM Tris, 10 mM dithiothreitol, pH 8.5 and purified to homogeneity by anion-exchange chromatography on a Mono Q column (Amersham Biosciences, Freiburg, Germany). For biosensor measurements the protein was dialyzed against 50 mM Tris/HCl, pH 8.0, 10 mM dithiothreitol, 0.05% Chaps. Purity of the recombinant protein was monitored by SDS-PAGE.

RESULTS

SHP2 Contributes to the Attenuation of Interleukin-6 Signal Transduction—The cytoplasmic tyrosine 759 of the signal transducer gp130 mediates attenuation of IL-6 signaling (14–17). The activated phosphotyrosine 759 functions as a reporter site for the tyrosine phosphatase SHP2 (10) and for SOCS3 (35, 36). To determine whether SHP2 contributes to IL-6 signal attenuation, we analyzed IL-6-induced STAT activation and promoter induction in murine fibroblasts lacking exon 3 of SHP2. These cells express a mutant SHP2 protein lacking 65 amino acids within the N-terminal SH2 domain (48). We checked whether this mutation affects recruitment to the tyrosine motif 759 of gp130. Therefore, we analyzed binding of SHP2 to corresponding un-phosphorylated (resembling non-activated receptors) or phosphorylated (resembling activated receptors) gp130 receptor peptides in wild-type fibroblasts as well as in SHP2-mut cells. Cellular extracts of both types of cells were incubated with the indicated biotin-conjugated peptides. Peptide-protein complexes were precipitated with NeutrAvidin-coupled agarose and analyzed for presence of SHP2 by Western blotting (Fig. 1A). Essentially no SHP2 binding was found in extracts of SHP2 mut cells (left lanes), whereas SHP2 binding to the Y(p)759-peptide was apparent in the wild-type cells (right lane).

To compare STAT activation in response to IL-6 these cells (SHP2 mut) and corresponding wild-type fibroblasts (SHP2 wt) were stimulated with IL-6–IL-6R complexes. STAT3 DNA binding was analyzed by EMSA. Stimulation of cells expressing mutated SHP2 led to enhanced and sustained STAT3-DNA binding activity when compared with wild-type cells (Fig. 1B). We also tested whether the lack of wild-type SHP2 affects the activation of a STAT3-responsive promoter/reporter construct. Wild-type and mutant fibroblasts were transfected with a chimeric EpOR/gp130 receptor construct, which allows stimulation independently of endogenous gp130. In line with the data above, activation of a promoter construct comprising STAT3-binding sites fused to the luciferase reporter gene cDNA was enhanced in cells expressing mutated SHP2 (Fig. 1C). Mutated cells reconstituted by stable expression of wild-type SHP2 did not show enhanced reporter gene induction, similar to wild-type cells. These data clearly indicate that a functional SHP2 attenuates IL-6-signal transduction.

Additionally, we analyzed whether expression of a catalytically inactive form of SHP2 affects IL-6-mediated activation of downstream signaling components. Mutation of the conserved asparagine (Asp-425) in the catalytic domain of SHP2 is known to abolish enzymatic activity (49). Myc-tagged SHP2 or SHP2ΔA were expressed in COS-7 cells together with an expression vector for Jak1 and chimeric receptors containing the extracellular domain of the IL-5Rα or IL-5Rβ and the transmembrane and cytoplasmic domains of gp130. These receptor chimeras allowed us to study the IL-6 signal transduction independently from endogenous gp130 (17). After stimulating the cells with IL-5, lysates were prepared and immunoprecipitations with antibodies to IL-5Rβ, Jak1, or the Myc-tag of SHP2 and SHP2ΔA were carried out. The phosphorylation status of the precipitated proteins was detected in Western blots with phosphotyrosine-specific antibodies (Fig. 2A). As shown in the upper panel of Fig. 2A, stimulation of COS cells expressing wild-type SHP2 led to increased Jak1, IL-5Rβ, gp130, and SHP2 tyrosine phosphorylation. The expression of SHP2ΔA further increased the induced as well as the basal phosphorylation of these signaling molecules. Finally, we observed, that a high percentage of phosphorylated Jak1 coprecipitates with SHP2ΔA, which may indicate Jak1 as a direct substrate for SHP2.

SHP2 and SHP2ΔA and another catalytically inactive mutant SHP2C–S were also analyzed for counteracting IL-6-induced STAT3 tyrosine phosphorylation (Fig. 2B) and activation of the liver-specific, STAT3-dependent α2-macroglobulin promoter (Fig. 2C). SHP2, SHP2ΔA, and SHP2ΔS were expressed in COS-7 cells similarly as described for Fig. 2A. The

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Fig. 1. SHP2 is involved in negative regulation of IL-6 signal transduction. A, cellular extracts of murine embryonic 3T3 fibroblast from SHP2-Exon 3 (SHP2 mut)-deficient mice or corresponding wild-type cells (SHP2 wt) were incubated with biotinylated peptides Tyr-759 or Yip759 corresponding to the SHP2-binding site in gp130. After precipitation with NeutAvidin-coupled agarose the proteins were sub-

ected to Western blot analyses (WB) using antibodies to SHP2, B, STAT DNA binding in SHP2 wt and SHP2 mut cells after stimulation with 2 ng/ml IL-6 and 1 µg/ml sIL-6R for times indicated was analyzed by EMSA with STAT3-specific DNA probes. Bands resulting from STAT3 homodimers bound to the DNA probe are indicated by an arrowhead. C, wild-type cells, SHP2 mut cells and corresponding SHP2-reconstituted cells were transiently transfected with expression vectors coding for chimeric Epo/gp130 receptors (containing the extracellular part of the EpoR and the transmembrane and cytoplasmic part of gp130) and the SIE-tk-luciferase reporter gene construct (3 µg). The cells were stimulated for 16 h with 7 units/ml Epo as indicated, and cellular extracts were prepared for the determination of luciferase activity. Luciferase expression was normalized to the luciferase activity in cell extracts from stimulated wild-type cells. Data shown are the averages of triplicate independent determinations of firefly luciferase activity normalized to β-galactosidase activity ± S.E. and are representative of multiple similar experiments.

**TARGETED SHP2 RESTORES THE INHIBITORY ACTIVITY OF GP130 LACKING THE INHIBITORY TYROSINE 759**—The recruitment of SHP2 to phosphotyrosine peptides is known to activate its phosphatase activity (50). The underlying molecular mechanism was clarified by the three-dimensional structure of SHP2, which showed that the SH2 domains of inactive SHP2 cover the catalytic domain, whereas binding to phosphopeptides leads to a conformational change and activation of the enzyme (20). Deletion of the SH2 domains results in the formation of an active phosphatase, which cannot be recruited to phosphotyrosine motifs of activated receptors (23, 51). Signal transduction from gp130 containing a mutated tyrosine 759 is enhanced, because the receptor lacks the inhibitory SHP2-SOCS3 recruitment site. We have shown that the inhibitory tyrosine 759 of gp130 does not have to be located on the same receptor chain as the STAT-binding sites to exert its inhibitory action (17).

We asked whether forced recruitment of SHP2 to Y759F-mutated gp130 could restore the inhibitory activity in IL-6-induced signal transduction. Therefore, we fused the C-terminal part of SHP2 containing the tyrosine phosphatase domain (PTP) to gp130 lacking tyrosine 759. To maintain STAT activation, the C-terminal part of SHP2 was fused only to a single receptor chain of the gp130 dimer. Again, we used combinations of IL-5R/gp130 chimeric receptors, which allowed the heterodimerization of different cytoplasmic domains of gp130 through stimulation with IL-5. Flow cytometric analyses were performed to monitor whether mutations in the chimeric IL-5R/gp130 receptors affected cell surface expression. Whereas the mutation of single tyrosine residues in gp130 did not alter receptor expression significantly (Fig. 3A, flow cytometric profiles on the left panel), deletion of the 153 C-terminal amino acids of gp130 led to an enhanced surface expression (right panel), probably through the loss of the internalization signal in gp130 as previously observed (52–54). As shown in Fig. 3B, mutation of Tyr-759 ((IL-5R/gp130(YYYYYY)) —IL-5R/gp130(YFYYYY)) led to an increased promoter activity (compare 1 and 2). Tyrosine 759 also inhibits reporter expression when located in trans to the STAT recruitment sites on the second receptor chain ((IL-5R/gp130(YYYYYF)) —IL-5R/gp130(YFYYYYY)) (compare 3 and 4). Analysis of reporter gene induction through (IL-5R/gp130(YF)) —IL-5R/gp130(YFYYYYY)) (panel 5) shows that the C-terminal part of (IL-5R/gp130(YYYYYF)) (panel 4) does not contribute to signal transduction.

Interestingly, signal transduction through a receptor complex containing a receptor-fused SHP2-PTP domain was reduced ((IL-5R/gp130(YF)/PTP —IL-5R/gp130(YFYYYYY)) (compare 5 and 6) despite the higher expression of this fusion protein. In summary, receptor-targeted SHP2-phosphatase activity, at least partially, restores the inhibitory activity of a mutated tyrosine 759 motif in gp130.

The potential of the SHP2-PTP domain to restore the inhibitory activity of gp130 mutants lacking Tyr-759 should be confirmed with an independent experimental approach. Therefore, we checked whether a membrane-anchored SHP2-PTP domain also restores the inhibitory activity. For this purpose, the short membrane-anchoring signal of the Sre-kinase hck was fused to...
**Fig. 2.** SHP2 is involved in negative regulation of IL-6 signal transduction. A, COS-7 cells were cotransfected with expression vectors for IL-5Rα/gp130 and IL-5Rβ/gp130 (6 µg each), Jak1 (0.8 µg), and SHP2 or SHP2D→A (12 µg). 40 h after transfection the cells were starved with DMEM without FCS for 8 h. Cells were stimulated for 20 min with 10 ng/ml IL-5, and cellular extracts were prepared. Lysates were incubated with indicated antibodies and protein-antibody complexes were separated by SDS-PAGE and analyzed by Western blotting with the antibodies for activated STAT3 (upper panel) and after stripping of the blot with antibodies for STAT3 as a loading control. B, COS-7 cells were transduced with expression vectors for SHP2, SHP2D→A, or SHP2C→S (10 µg), IL-5Rα/gp130 and IL-5Rβ/gp130 (6 µg each), Jak1 (0.8 µg), and STAT3 (3 µg) 40 h after transfection. Cells were stimulated with 80 ng/ml IL-5 of left untreated. 30 min post stimulation cells were harvested, and lysates were prepared. The same amounts of protein (130 µg) were separated by SDS-PAGE and analyzed by Western blotting with the antibodies for STAT3 (upper panel) and after stripping of the blot with antibodies for STAT3 as a loading control. C, HepG2 cells were cotransfected with expression vectors encoding EpoR/gp130 chimeric receptors (2 µg), the α,β-M-215 luciferase reporter-gene construct (5 µg), and SHP2, SHP2C→S, or SHP2D→A (4 µg). The cells were stimulated for 16 h with 4 units of Epo/ml as indicated, and cellular extracts were prepared for the determination of luciferase activity. Luciferase expression was normalized to the luciferase activity in cell extracts from stimulated cells transfected with wild-type SHP2. Data are given as means ± S.E. of at least three independent experiments.
FIG. 3. Targeted SHP2 restores the inhibitory activity of gp130 mutants lacking the inhibitory tyrosine 759.

A, surface expression of the IL-5Rα/gp130 chimeras and IL-5Rα(YF-PTP) fusion proteins was monitored by FACS analyses in transiently transfected COS-7 cells, using an antibody raised against the extracellular part of the IL-5Rα (open histograms). Filled histograms show FACS controls with secondary antibody only. B, human hepatoma cells (HepG2) were transfected with equal amounts (2 μg) of expression vectors coding for chimeric receptors as indicated in the figure and an αM-promoter-luciferase reporter construct (pGL3αM-215Luc). The cells were stimulated for 16 h with 10 ng/ml human recombinant IL-5 where indicated. Cellular extracts were prepared for determination of luciferase activity, which was normalized to the activity of coexpressed β-galactosidase. Luciferase activity is presented in relation to the activity in cellular extracts from stimulated HepG2 cells expressing the IL-5Rα- and IL-5Rβ-gp130 wild-type chimeric receptors. Data are given as means ± S.D. of at least three independent experiments. C, HepG2 cells were transiently transfected with 20 μg of expression vectors for PTP, Ψ-PTP, or the corresponding control plasmid. 48 h post transfection cells were harvested, and proteins were separated by SDS-PAGE. Western blots were developed with SHP2 antibodies. D, HepG2 cells were transfected with 2 μg of expression vectors for the EpoR/gp130 chimeric proteins EG(YYYYYY) or EG(YFYYYY) together with 4 μg of expression vectors for PTP, Ψ-PTP, or the corresponding control vector and the αM-promoter-luciferase reporter construct. Cells were stimulated for 16 h with 7 units/ml Epo, lysed, and assayed for luciferase activity as described above.
we prepared SOCS3 deletion mutants, lacking the C-terminal SOCS-box (S3Δbox), the 23 N-terminal amino acids (S3ΔN-term), or, in addition to the latter, a region identified as an extension of the SH2 domain (28, 30) (S3ΔESS) (Fig. 5A). These proteins were transiently expressed in COS-7 cells (Fig. 5B) and tested for SHP2 binding using coimmunoprecipitation assays (Fig. 5D). As a control, binding of these proteins to the phosphotyrosine peptide Y(p)759 of gp130 was monitored by peptide precipitation assays (Fig. 5C). Fig. 5C shows that neither the SOCS-box nor the N-terminal 23 amino acids of SOCS3 is essential for binding to Y(p)759. However, the extended SH2 domain (ESS) of SOCS3 is essential for binding to the receptor peptide Y(p)759. Binding to SHP2 appeared to be independent of the ESS region, because none of these mutations impaired binding to SHP2 (Fig. 5D, upper panel). Another set of SOCS3 mutants with C- and N-terminal deletions or single point mutations was used to further map the region responsible for SHP2-binding (Fig. 5E). Deletion of the N-terminal 36 amino acids (S3ΔN36) as well as point mutations within the ESS (S3L41R) and within the SH2 domain (S3R71K; S3R71E) of SOCS3 impaired binding to the receptor peptide (Fig. 5F, upper panel). Only the latter mutant within the SOCS3-SH2 domain impaired SOCS3/SHP2 complex formation (Fig. 5F, lower panel). These data demonstrate that binding of SOCS3 to gp130 or to SHP2 depends on the integrity of the SOCS3-SH2 domain. Different modes of interaction have to be assumed, because binding of SOCS3 to the receptor peptide is affected by the exchange of Arg-71 to Lys but binding to SHP2 is only impaired by exchange to Gln. Additionally, these experiments demonstrate that receptor binding requires the ESS of SOCS3, because deletion of the ESS as well as a single point mutation (L41R) within the ESS of SOCS3 impairs its binding to the gp130 receptor peptide but not to SHP2.

SOCS3 Interacts with the Y(p)542 Module of SHP2—The SHP2-SOCS3-binding site Y(p)759 of gp130 (VQpYSTVH) and the Y(p)542 motif of SHP2 (HEpYNTIK) display some structural and functional similarities. SHP2 binds to the tyrosine motif of gp130 (10), additionally, the N-terminal SH2 domain of SHP2 interacts intramolecularly with the phosphotyrosine 542 motif of SHP2 to overcome autoinhibition (21). SOCS3 also binds to the Y(p)759 of gp130 (35, 36). Thus, we speculated that SOCS3 may bind SHP2 through the Y(p)542 motif of SHP2. Indeed, peptide precipitations in Fig. 6A show that SOCS3 binds both the Y(p)759-peptide, derived from gp130 and the Y(p)542-peptide from SHP2, but not the non-phosphorylated Tyr-542-peptide or unrelated peptides from gp130 (Y(p)683) or SHP2 (Y580 and Y(p)580). To further compare binding of SOCS3 to the gp130 and SHP2 peptides, biosensor analyses were performed with recombinant human SOCS3 thioridoxin fusion proteins. Fig. 6B shows that SOCS3 specifically binds the Y(p)759 motif of gp130 and the Y(p)542 peptide of SHP2. Hardly any binding could be detected using the Y(p)580 of SHP2 or the unphosphorylated Tyr-542 peptide. To compare the dissociation constants for the binding of SOCS3 to Y(p)542 of SHP2 and to Y(p)759 of gp130, binding was analyzed in the presence of decreasing amounts of SOCS3 (Fig. 6, C and E, respectively). $K_D$ values derived from Scatchard plots (Fig. 6, D and F) were 210 nM for the SOCS3/Y(p)759 interaction and 3.5 $\mu M$ for binding the Y(p)542 peptide of SHP2. Thus, the affinity of SOCS3 to the receptor peptide appears to be 17 times higher than to the SHP2-peptide. Nevertheless, a remarkable affinity was found for the binding of SOCS3 to the phosphotyrosine 542 motif of SHP2.

SHP2 Targeting to Receptors Lacking the SHP2-SOCS3 Recruitment Site Tyr-759 Does Not Restore Sensitivity to SOCS3—Mutation of Y759F in gp130 affects gp130-dependent promoter activation as well as SHP2 recruitment (10, 14–17). Furthermore, gp130 mutated at Tyr-759 is not sensitive to inhibition by SOCS3 and does not bind SOCS3 (35, 36). We asked whether targeted SHP2 also restores sensitivity to SOCS3 in receptor complexes lacking the SHP2-SOCS3 recruitment site.

The effect of SOCS3 expression on $\alpha_M$-promoter induction by activated chimeric receptors was analyzed (Fig. 7A). Expression of SOCS3 inhibited signal transduction through receptor complexes containing the cytoplasmic part of wild-type gp130 (IL-5Rα/gp130(YYYYY) · IL-5Rβ/gp130(YYYYY)) (compare 1 and 2 in Fig. 7A). In contrast, activation of chimeric receptors lacking Tyr-759 (IL-5Rα/gp130(YFYYYY) · IL-5Rβ/gp130(YFYYYY)) led to promoter induction (3). 2M-promoter induction was measured in receptor complexes expressing gp130(YYYYY) · IL-5Rβ/gp130(YYYYY) and after cotransfection with expression vectors coding for EG(YYYYY) or SOCS3-cDNA where indicated and 2 $\mu g$ of SOCS3-cDNA where indicated and 2 $\mu g$ of a SIE-tk-luciferase reporter gene. The cells were stimulated for 16 h with 7 units/ml Epo as indicated, and cellular extracts were prepared for the determination of luciferase activity. Luciferase expression was normalized to the luciferase activity in cell extracts from stimulated cells expressing EG(YYYYYYY). Data are given as means ± S.E. of at least three independent experiments.
(YFYYYY) caused enhanced reporter activity and is not sensitive to SOCS3 (compare 3 and 4 with 1 and 2). Targeting the C terminus of wild-type SHP2 to the receptor complex attenuated signal transduction (compare 6 and 7). The comparison of 7 and 8 shows that SOCS3 hardly affects signal transduction through these receptors.
Similar results were obtained with membrane-anchored SHP2 (Fig. 7B). Signal transduction through chimeric EpoR/gp130 lacking Tyr-759 is not affected by coexpression of SOCS3 binding to different SHP2 phosphotyrosine motifs. Biotinylated peptides encompassing tyrosine motifs of SHP2 and gp130 were immobilized on SA chips and the association with SOCS3 was measured by means of surface plasmon resonance. The concentration of SOCS3 in this experiment was 15 μM. Nonspecific binding was determined by using unphosphorylated peptides as well as purified thiodoxin solution (TRX, 3.5 μM). A phosphorylated peptide encompassing Tyr-759 of gp130 served as a positive control. C–F, quantitative comparison of SOCS3 binding to SHP2- and gp130-peptides. SOCS3 was diluted 2-fold from 34 μM to 17 nM (Y(p)542) or 235 nM (Y(p)759), and the interaction with the phosphopeptides was measured. Plateau values of the binding curves were taken for calculation of the K_d values. Graphs used for determination of the affinity constants for peptides were Y(p)542 (C and D) and Y(p)759 (E and F). C and E, sensorgram showing the interaction of serial dilutions of SOCS3 and the phosphopeptides Y(p)542 and Y(p)759, respectively. D and F, Scatchard analyses of the association of SOCS3 with peptides Y(p)542 and Y(p)759, respectively. Shown is one out of three sets of experiments, which gave similar results.

Similar results were obtained with membrane-anchored SHP2 (Fig. 7B). Signal transduction through chimeric EpoR/gp130 lacking Tyr-759 is not affected by coexpression of SOCS3 but by coexpression of membrane-anchored SHP2 (Ψ-PTP) (compare 3 and 4 with 3 and 5 in Fig. 7B). The presence of membrane-targeted Ψ-PTP did not restore SOCS3 inhibitory activity on receptors lacking the SOCS3-SHP2 recruitment site (compare 5 and 6). Expression of non-targeted SHP2-PTP domains (PTP) did not affect signaling through EpoR/gp130(YFYYYY) as already shown above.

From these data we suggest that targeted SHP2 can not restore inhibitory activity of SOCS3 on gp130-(Y759F)-mutated receptors suggesting that the interaction of SHP2 with SOCS3 is not involved in signal attenuation. Thus, the physiological relevance of this interaction has to be clarified by further experiments.

Modulation of ERK Activity through SOCS3 Mutants—Tyrosine 759 of gp130 is also essential for the activation of the MAPK cascade. Mutation of tyrosine 759 to phenylalanine was shown to abolish IL-6-mediated activation of Erk1/Erk2 (55). It has been suggested that recruitment of SHP2 to this phosphotyrosine 759 motif mediates activation of Erk1/Erk2. Thus, one potential function of the SHP2-SOCS3 complex might be to modulate IL-6-induced MAPK activity. Therefore, SOCS3 mutants with different potential to bind gp130 and SHP2 were
hances IL-6 signal transduction led to the assumption that SHP2 mediates an inhibitory activity (14–16, 18). The contribution of SHP2 to tyrosine 759-dependent signal attenuation had to be re-examined, because we found out that SOCS3 also acts by binding to the phosphotyrosnine 759 motif of gp130 (35). This observation was confirmed by affinity measurements showing a higher affinity of SOCS3 to the phosphorylated Tyr-759 receptor peptide than to peptides corresponding to the activation loop of the Janus kinases Jak1, 2, or 3 (36).

Here we demonstrate that SHP2 indeed affects IL-6 signaling, because stimulation of cells lacking full-length SHP2 results in an increased gp100-mediated signal transduction as measured by STAT activation and STAT3-dependent gene induction (Fig. 1). In addition, expression of a catalytically inactive form of SHP2 leads to an increase in receptor, Jak, and STAT3 phosphorylation, which could neither be precipitated with the receptor mutants nor with SHP2, did not act inhibitory on gp130 mutants lacking the SOCS3/SHP2 recruitment site in gp130 can be restored by membrane targeting of the C-terminal part of SHP2 containing the PTP domain and also by fusion of this polypeptide to the membrane-proximal part of gp130 (Fig. 3). These data indicate an inhibitory function of SHP2 on IL-6 signaling independent from receptor-bound SOCS3. On the other hand, SOCS3 action does not depend on functional SHP2, because cells expressing only mutated SHP2 remain sensitive to SOCS3, but become insensitive, when the SOCS3/SHP2 recruitment site in gp130 is mutated (Fig. 4).

We observed that SOCS3 binds in a different manner to receptor peptides and SHP2 (Fig. 5). Binding to tyrosine 759 of gp130 was highly sensitive to mutations within the ESS and the SH2 domain of SOCS3. In clear contrast, only the substitution of the crucial arginine within the SH2 domain of SOCS3 by glutamate impaired binding of SOCS3 to SHP2. Furthermore, SOCS3 binds the phosphotyrosine peptide derived from the Tyr-759 motif of gp130 with a 17-fold higher affinity than the peptide derived from the Tyr-542 motif of SHP2 (Fig. 6). These affinities may also reflect the more restrictive requirements for binding to gp130. Whether this interaction is really of major relevance for SOCS3 function remains to be further elucidated.

From all these data, we suggest that there are two, largely distinct modes of negative regulation of gp130 activity, and probably of other cytokine receptors, despite the fact that both SOCS3 and SHP2 are recruited to the same site within gp130. Binding to the same receptor site implies competitive binding of both proteins to gp130, which would require SHP2 and SOCS3 being present at the receptor at the same time. This seems to be possible, because SOCS3 protein becomes detectable already after 20 min, at a time where SHP2 is still phosphorylated (data not shown). Tyrosine phosphorylation was recently clearly linked to enzymatic activity of SHP2 (21). Because SOCS3 protein remains longer detectable than SHP2 phosphorylation, SHP2 may be important for early signal modulation, whereas SOCS3 has to be induced and probably attenuates signaling subsequently. Furthermore, it is intriguing to speculate in terms of cross-talk, assuming that SOCS3 may also be induced by other cytokines prior to IL-6-stimulation. As a consequence SOCS3 would be immediately available to block IL-6 signaling and also to compete with SHP2 for receptor binding.

Similar to our results for the signal transduction of IL-6, it has been shown that SOCS3 and SHP2 are recruited to the same receptor motifs within the leptin receptor. Furthermore, the inhibitory function of SOCS3 depends on this SOCS2/SHP2 binding site (56, 57). In contrast to the data for IL-6 signaling presented here, it has been suggested that SOCS3 but not SHP2 is involved in attenuation of leptin signaling. Instead, SHP2 was attributed to mediate MAPK activation (56, 58).

**DISCUSSION**

After stimulation with IL-6, the protein-tyrosine phosphatase SHP2 is recruited to the phosphotyrosine motif 759 of gp130 within the activated receptor complex (10). The observation that the mutation of tyrosine 759 to phenylalanine en-
Tyrosine 759 of gp130 is also essential for the activation of the MAPK cascade (55). The current view of this activation, adopted from epidermal growth factor and platelet-derived growth factor signal transduction, is that SHP2 links the Grb2/SOS complex and/or Gab1/2 to gp130 (55, 59, 60). Additionally, it has been found that IL-6-induced association of the adapter protein Gab1 with SHP2 also leads to an activation of the MAPK cascade (61). Recent data in this issue by Calanciu et al. (62) suggested that the MAPK cascade can also be affected through SOCS3 in response to IL-2, Epo, and platelet-derived growth factor. SOCS3 becomes tyrosine phosphorlated and subsequently binds and inactivates Ras/GAP.

The latter leads to sustained Ras activation and consequently to activation of the MAPK cascade. We presented the first evidence that SOCS3 might also modulate MAPK activation after stimulation with IL-6. It is further intriguing to speculate that SOCS3 may specifically inhibit the Jak/STAT pathway while maintaining MAPK activation.

Meanwhile, several other cytokine and growth factor receptors have been described to bind SOCS3 as well as SHP2 (47, 56). For each of these receptors the individual role of SHP2 and SOCS3 in IL-6 signal attenuation has to be elucidated. For the Jak/STAT pathway, the role of SOCS3 in IL-6 signal attenuation was recently described (48).

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Note Added in Proof—Bartoe and Nathanson pointed out that LIF signaling is also independently and negatively regulated by SHP2 and SOCS3 (Bartoe, J. L., and Nathanson, N. M. (2002) Mol. Brain. Res. 107, 108–119).

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