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Suppressor of Cytokine Signaling-3 Is Recruited to the Activated Granulocyte-Colony Stimulating Factor Receptor and Modulates its Signal Transduction

Michael Hörtner,*† Ulrich Nielsch,* Lorenz M. Mayr,‡ James A. Johnston,§ Peter C. Heinrich,‡*† and Serge Haan‡

G-CSF is a polypeptide growth factor used in treatment following chemotherapy. G-CSF regulates granulopoiesis and acts on its target cells by inducing homodimerization of the G-CSFR, thereby activating intracellular signaling cascades. The G-CSFR encompasses four tyrosine motifs on its cytoplasmic tail that have been shown to recruit a number of regulatory proteins. Suppressor of cytokine signaling 3 (SOCS-3), also referred to as cytokine-inducible Src homology 2-containing protein 3, is a member of a recently discovered family of feedback inhibitors that have been shown to inhibit the Janus kinase/STAT pathway. In this study, we demonstrate that human SOCS-3 is rapidly induced by G-CSF in polymorphonuclear neutrophils as well as in the myeloid precursor cell line U937 and that SOCS-3 negatively regulates G-CSFR-mediated STAT activation. Most importantly, we show that SOCS-3 is recruited to the G-CSFR in a phosphorylation-dependent manner and we identify phosphotyrosine (pY)729 as the major recruitment site for SOCS-3. Furthermore, we demonstrate that SOCS-3 directly binds to this pY motif. Surface plasmon resonance analysis reveals a dissociation constant (K_d) for this interaction of around 2.8 μM. These findings strongly suggest that the recruitment of SOCS-3 to pY729 is important for the modulation of G-CSF-mediated signal transduction by SOCS-3. The Journal of Immunology, 2002, 169: 1219–1227.

G granulocyte colony-stimulating factor is a cytokine that plays an important role in hematopoietic processes by stimulating the proliferation, the differentiation, and the survival of committed myeloid progenitors (for reviews, see Refs. 1 and 2). As a major regulator of neutrophil production (3, 4), it is commonly used in the treatment of neutropenia following chemotherapy (5) and of severe congenital neutropenia (SCN)3 (6). G-CSF exerts its biological effects through the interaction with its cell surface receptor, G-CSFR. A correlation between the acquisition of G-CSFR mutations and the leukemic progression of SCN (estimated at 10%; Refs. 7 and 8) has been observed (9–11).

G-CSF has been shown to specifically activate Janus kinase (JAK)1, JAK2, and TYK2 as well as the transcription factors STAT1, STAT3, and STAT5 (12–16). STAT3 activation has been estimated at 10%; Refs. 7 and 8) has been observed (9 –11).

Suppressor of cytokine signaling 3 (SOCS-3), also referred to as cytokine-inducible Src homology 2-containing protein 3, is a member of a recently discovered family of feedback inhibitors that have been shown to inhibit the Janus kinase/STAT pathway. In this study, we demonstrate that human SOCS-3 is rapidly induced by G-CSF in polymorphonuclear neutrophils as well as in the myeloid precursor cell line U937 and that SOCS-3 negatively regulates G-CSFR-mediated STAT activation. Most importantly, we show that SOCS-3 is recruited to the G-CSFR in a phosphorylation-dependent manner and we identify phosphotyrosine (pY)729 as the major recruitment site for SOCS-3. Furthermore, we demonstrate that SOCS-3 directly binds to this pY motif. Surface plasmon resonance analysis reveals a dissociation constant (K_d) for this interaction of around 2.8 μM. These findings strongly suggest that the recruitment of SOCS-3 to pY729 is important for the modulation of G-CSF-mediated signal transduction by SOCS-3. The Journal of Immunology, 2002, 169: 1219–1227.

Materials and Methods

Materials

Biotinylated peptides were purchased from Jerini Biotools (Berlin, Germany). The respective sequences of the phosphorylated and unphosphorylated G-CSFR peptides used in this study were: PTLVQTpYVLQGDP (pY704), TSDQVLpYPQGLLS (pY729), SPGPHpYLRCDST (pY744), TPSKPSpYENLWFQ (pY764), PTLVQTpYVLQGDP (pY704), TSDQVLpYPQGLLS (pY729), SPGPHpYLRCDST (pY744), TPSKPSpYENLWFQ (pY764).
Cell culture medium, fetal calf serum, and other media supplements were obtained from Life Technologies (Rockville, MD). Restriction enzymes were exclusively from NEB (Frankfurt, Germany). Zeocin was purchased from Invitrogen (Karlsruhe, Germany).

**Cells and culture conditions**

Human embryonic kidney (HEK293/CRL-1573) cells were obtained from American Type Culture Collection (Manassas, VA). PG13 (33) packaging cells were used for experiments with the agreement of H. Hanenberg, University of Düsseldorf, (Düsseldorf, Germany). U937 cells were obtained from American Type Culture Collection. HEK293 and PG13 packaging cells were grown in DMEM. U937 cells were cultured in RPMI medium. All media were supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C, 5% CO₂. For stimulation, cells were treated with 50 ng/ml human rG-CSF (Amgen, Thousand Oaks, CA) and harvested at the time points indicated. Transduced cells were additionally cultured with 10 μg/ml zeocin.

**Plasmids**

The retroviral vector pMSCVneo (Clontech Laboratories, Heidelberg, Germany) was digested with EcoRI. Then, a PCR-amplified fragment containing the multiple cloning site (MCS) of BlueScriptII KS(+) (Stratagene, Heidelberg, Germany) was digested with ApoI/EcoRI and ligated into pMSCVneo. Primers flanking the MCS of BlueScriptII KS(+) were 5’-TTTA AAATTTGAATTTGAGCTCCACCGCGGTGGCGGCCGC-3’ and 5’-TTATTA TTTAATTTGTTGATCCCCGGGCTGACGAATTC-3’, respectively. pMSCVneo was then digested with BglII and a PCR-amplified fragment comprising an internal ribosome entry site-enhanced GFP sequence was ligated between BamHI/AscI into the MSCV backbone. PCR primers for the IRES-EGFP-ZEO fragment were as follows: 5’-TTTAATTTGAATTTGAGCTCCACCGCGGTGGCGGCCGC-3’ and 5’-TTATTA TTTAATTTGTTGATCCCCGGGCTGACGAATTC-3’, respectively. The tagging vector pMYZ404. Human SOCS-3 cDNA was amplified from EST no. 725896 (Research Genetics, Huntsville, AL) and cloned into pMYZ404 (pMYZ404-SOCS-3). Human SOCS-1 was PCR-amplified from a human lymph node cDNA library (Invitrogen, Karlsruhe, Germany) and subcloned into pcDNA 4.1 HisMax (Invitrogen). The SOCS-2 construct was kindly provided by Dr. A. Yoshimura (Kyushu University, Fukuoka, Japan). Human G-CSF and STAT response element-luciferase cDNAs were a kind gift from Dr. A. Wilmen (Bayer, Wuppertal, Germany). The STAT response element consisted of six identical repeats of the STAT1, STAT3, STAT4, and STAT5 A DNA binding motif TTCTnGAA (34), spaced by 14 nucleotides, respectively. Y704F and Y729F G-CSF mutants were generated using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutagenesis primers for the Y704F as well as the Y729F mutant were as follows: 5’-GCCACCTCTGTCATCGACACCTGTGTTCTGGGGGACCC-3’ (Y704F sense), 5’-GGGCTCCCCTTCGAAGCCACAAAGGCTTGAC CAGATGTC-3’ (Y704F antisense), 5’-GGGACACCGAGGATCTG CACCTTTGGCCGACGCTGGCAGGCCC-3’ (Y729F sense), and 5’- GGGCTCCGCCAGCAGTCTGGCACAAGAGGACCTGATCGTGGTA CC-3’ (Y729F antisense).

**Transfection and retroviral transduction of HEK293 cells**

HEK293 cells were transfected with 5 μg of human G-CSFR and the luciferase reporter construct under the control of the STAT response element-luciferase cDNA using transfection reagents (Life Technologies); 48 h after transfection, single clone selection was performed. After 3 wk, a number of clones stably carrying the cDNAs were expanded. The clones that responded best to G-CSF stimulation were taken for the experiments. For transient expression of SOCS-1 and SOCS-3, HEK293 cells were transfected with the indicated amount of cDNA using the fuGENE6 (Roche, Mannheim, Germany) transfection reagent.

Stable PG13 producer cells containing pMYZ404 and pMYZ404-SOCS-3 were grown to 100% confluency. Then, medium was exchanged and 1 day later retroviral supernatant was collected, filtered, and stored at −70°C until used. HEK293/G-CSFR/STAT/luciferase cells (1 × 10⁵) were seeded into six-well plates, incubated overnight, and then infected with the retroviral supernatants in the presence of 5 μg/ml protamine. After 24 h of infection, media were replaced, and after 48 h of cultivation, cells were selected with 50 μg/ml zeocin. The transduction efficiency was assessed by fluorescence microscopy. After 2 wk of selection, the cells were analyzed and used for experiments.

RT-PCR analysis of human polymorphonuclear neutrophils (PMN) and U937 cells for SOCS-3 expression

Blood samples were layered onto Ficoll-Hypaque, and PMN were isolated by centrifugation and hypotonic lysis of erythrocytes. Cells were rested in serum-free medium for 1 h (PMN) or 2 h (U937 cell line), and then stimulated with 50 ng/ml G-CSF for the times indicated. Total RNA was isolated from PMN (5 × 10⁵ cells/RT-PCR) or cultured U937 (10 × 10⁵ cells/RT-PCR) cells using the RNA STAT-60 method (Biogenesis, Poole, U.K.) following the manufacturer’s instructions. Total RNA was performed using 1 μg of total cell mRNA using the OneStep RT-PCR kit from Qiagen (Hilden, Germany). PCR amplification was performed using primer pairs specific for human CIS (upstream primer, 5’-GATCTGCTGTGATCACGCCA-3’; downstream primer, 5’-ACAAGGCGCTCACAGGTATT-3’), SOCS-1 (upstream primer, 5’-GAGAGGCTCTGACGCTCTT-3’; downstream primer, 5’-GGATAGCCAGGCCATGGCA-3’), SOCS-2 (upstream primer, 5’-GATAAGCGGACAGCTTCA-3’; downstream primer, 5’-AA GAAGGCAGACTTCTGGA-3’), and GAPDH as a control (upstream primer, 5’-TGAAGATCAAGAGGTTG-3’; downstream primer, 5’-TTTCTAGAGG GACCATGGT-3’), the predicted products for CIS, SOCS-1, SOCS-2, SOCS-3, and GAPDH being 45, 562, 500, 554, and 244 bp, respectively. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

**STAT reporter gene assays and STAT activation in total cell lysates**

For reporter gene assays, HEK293/G-CSFR/STAT/luciferase cells were seeded on a 96-well microtiter plate at a density of 1 × 10⁶ cells/well. Cells were incubated overnight, stimulated with 0.2 pg/ml to 1 μg/ml human G-CSF and luciferase activity was measured after 6 h of stimulation.

To study the effect of SOCS-1 and SOCS-3 on STAT-activation, HEK293/G-CSFR/STAT/luciferase were transiently transfected with SOCS-1 or myc-tagged SOCS-1 constructs and stimulated with 50 ng/ml G-CSF for 10 min. Total cell lysates were prepared and analyzed by Western blot analysis. To examine the extent of STAT3 phosphorylation, the blot was detected with a specific phospho-STAT3 Ab (cell signaling) and then redetected with a specific STAT3 Ab (C20; Santa Cruz Biotechnol., Heidelberg, Germany). Expression of SOCS-1 and SOCS-3 was detected with a myc-Ab (9E10; Research Diagnostics, Flanders, NJ) or SOCS-3 Ab (M20; Santa Cruz Biotechnol., respectively).

**Pep tide and coimmunoprecipitation assays**

Approximately 0.15 μmol of the biotinylated peptides were immobilized by incubation with 2.5 mg streptavidin (SA) Sepharose (Amerham Pharmacia Biotech) was used to visualize the immunoreactive bands by ECL detection.
resolved by SDS-PAGE and visualized by Western blot techniques using Abs to SOCS-3 and G-CSFR.

Expression of SOCS-3 in bacteria

SOCS-3 was expressed as a thioeprxin fusion protein in BL21 (DE3) Escherichia coli (Stratagene). Bacteria were grown in Luria-Bertani medium containing 100 µg/ml ampicillin at 37°C to an A600 of 1.0 and then induced with 1 mM isopropyl-1-thio-β-galactopyranoside. Cells were harvested after 3 hr of expression, resuspended in 50 mM Tris-Cl, pH 8.0, 10% glycerol, and lysed by sonication. SOCS-3 was purified on a HiTrap chelating column (Amersham Pharmacia Biotech) with Nickel-IDA as the matrix. Native eluted SOCS-3 was dialyzed into 50 mM Tris-HCl, 10 mM DTT, pH 8.5, and purified to homogeneity by anion exchange chromatography on a MonoQ column (Amersham Pharmacia Biotech). For biosensor measurements, the protein was dialyzed against 50 mM Tris-HCl, pH 8.0, 10 mM DTT, 0.05% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate. Purity of the recombinant protein was monitored by SDS-PAGE.

Biosensor analysis

Biotinylated peptides were loaded on a SA-coated Biosensor chip (Biacore, Freiburg, Germany). The amount of loaded peptide was 80 ± 4 fmol/mm² chip surface which corresponds to 141 ± 5 response units. Before loading of the sensor chip with peptide, the surface was washed three times for 30 sec with 1 M NaCl in 50 mM NaOH. Peptides (100 ng/ml) were loaded onto the chip until 150 response units. Protein-peptide interactions were measured by injection of serial dilutions of SOCS-3 over the chip surface at a flow rate of 20 µl/min for 1 min. Before injection of SOCS protein, the sensor chip was flushed with BSA (0.1 mg/ml) at a flow rate of 20 µl/min for 1 min. For measurement of the KD value, the flow rate was enhanced to 100 µl/min to obtain a higher resolution of kinetics. For this type of experiment, SOCS-3 was injected for 4 min, dissociation time was 5 min, regeneration of the chip between the measurements in all experiments performed was done at 20 µl/min with 1 M NaCl in 50 mM NaOH for 30 s. Binding curves were analyzed by using the BiaEvaluation software 3.0.1 (Biacore). To correct for nonspecific binding events, an empty sensor surface without peptide was analyzed in parallel during protein injection. Additionally, thioredoxin was injected at high concentrations (3.5 M) to rule out nonspecific interactions of the fusion protein of SOCS-3. Curves were plotted with subtracted nonspecific binding. Determination of the dissociation constants was done by Scatchard analysis (35).

Results

G-CSF rapidly induces SOCS-3 in PMN and the monocyte precursor cell line U937

To investigate the role of SOCS-3 in G-CSF signaling, we first analyzed SOCS-3 mRNA induction in the human myeloid precursor cell line U937 that endogenously expresses the G-CSFR. SOCS-3 mRNA induction in these cells was monitored by RT-PCR. Fig. 1A shows that although SOCS-3 mRNA is already constitutively expressed in U937 cells, stimulation with G-CSF leads to an immediate increase in SOCS-3 mRNA with the expression peaking around 45 min after stimulation. The mRNA levels then gradually decline and return to nearly basal levels within 3 h. In parallel, we checked for the induction of other members of the SOCS family, namely CIS, SOCS-1, and SOCS-2. As shown in Fig. 1A, SOCS-1 is also induced by G-CSF, with mRNA levels peaking around 60 min after stimulation. CIS and SOCS-2 mRNA are not regulated by G-CSF. To further investigate whether G-CSF also induces SOCS-3 in freshly isolated PMN, the PMN fraction of healthy donors was stimulated with G-CSF. We observed that G-CSF also strongly induces SOCS-3 mRNA in these primary cells with similar induction kinetics as in U937 cells. As seen in U937 cells, G-CSF also induced SOCS-1 mRNA in neutrophils (Fig. 1B). SOCS-2 and CIS mRNA was not found to be regulated by G-CSF in these cells (data not shown).

SOCS-3 and SOCS-1 inhibit G-CSFR-mediated signal transduction

As we and others have observed that SOCS-3 acts on IL-6 signal transduction by binding to pY759 of gp130 (31, 32) and as the G-CSFR is closely related to gp130, it was important to determine whether there is a similar inhibitory mechanism mediated by SOCS-3 in the case of the G-CSFR. We first investigated the effect of SOCS-3 on G-CSF-induced signal transduction. For this purpose, HEK293 cells were stably transfected with cDNAs encoding G-CSFR and a STAT-luciferase response element responsive to STAT1, STAT3, and STAT5 and then retrovirally transduced with a construct coding either for green fluorescent protein (GFP) or SOCS-3. Cells were stimulated with G-CSF and luciferase activity was measured after 6 h. The dose-response curves in Fig. 2A clearly demonstrate the inhibitory effect of SOCS-3 on the STAT response causing a depression of the maximal response to G-CSF by 74%. The control experiment with cells transduced with a vector containing GFP alone gave a similar dose-response curve as the mock-transduced cells and the same maximal response was maintained.

As we found G-CSF to induce both SOCS-3 and SOCS-1, we also investigated the effect of SOCS-1 on G-CSF signal transduction. Fig. 2B shows that SOCS-1, like SOCS-3, impairs G-CSF-induced STAT3 phosphorylation. We also found SOCS-1 to inhibit the STAT-mediated induction of gene expression after G-CSF in a reporter gene assay (data not shown).

SOCS-3 is recruited to the G-CSF receptor upon G-CSF stimulation

SOCS-3 was initially shown to inhibit JAK/STAT signaling by binding to and inhibiting JAK (22–24). As we and others have found that SOCS-3 exerts at least part of its inhibitory action through binding to cytokine receptor pY motifs (31, 32, 36–39), we investigated whether SOCS-3 is also recruited to the G-CSFR.
To study possible interactions of SOCS-3 with the G-CSFR, co-immunoprecipitation experiments were performed in HEK293 cells stably transfected with G-CSFR and SOCS-3 or G-CSFR alone. Fig. 3, A and B, shows that both proteins can be readily coimmunoprecipitated. Cell lysates from stimulated and nonstimulated cells either transduced with human SOCS-3 or nontransduced were incubated with an Ab recognizing epitopes on the human G-CSFR. As shown in Fig. 3A, SOCS-3 associates with the G-CSFR in a stimulation-dependent manner. Phosphorylation of the G-CSFR was verified by probing the membrane with an anti-pY Ab. Similarly, Fig. 3B shows the coimmunoprecipitation of the G-CSFR with SOCS-3. Cell lysates of HEK/G-CSFR or HEK/G-CSFR/SOCS-3 cells were precipitated with an Ab raised against SOCS-3 and probed for tyrosine phosphorylation, G-CSFR, and SOCS-3. Coprecipitated G-CSFR could only be detected in cells stimulated with G-CSF whereas no G-CSFR was detected in nonstimulated cells as well as HEK293/G-CSFR cell lysates lacking SOCS-3. These data confirm the stimulation-dependent association of SOCS-3 with the G-CSFR.

To assess whether SOCS-3 is recruited to the G-CSFR under physiological conditions, we performed coimmunoprecipitation experiments in U937 cells transfected with human SOCS-3 or nontransduced cells either transduced with human SOCS-3 or nontransduced were incubated with an Ab recognizing epitopes on the human G-CSFR. As shown in Fig. 3A, SOCS-3 associates with the G-CSFR in a stimulation-dependent manner. Phosphorylation of the G-CSFR was verified by probing the membrane with an anti-pY Ab. Similarly, Fig. 3B shows the coimmunoprecipitation of the G-CSFR with SOCS-3. Cell lysates of HEK/G-CSFR or HEK/G-CSFR/SOCS-3 cells were precipitated with an Ab raised against SOCS-3 and probed for tyrosine phosphorylation, G-CSFR, and SOCS-3. Coprecipitated G-CSFR could only be detected in cells stimulated with G-CSF whereas no G-CSFR was detected in nonstimulated cells as well as HEK293/G-CSFR cell lysates lacking SOCS-3. These data confirm the stimulation-dependent association of SOCS-3 with the G-CSFR.

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motifs Y704, Y729, Y744, and Y764 of the human G-CSFR. The G-CSFR peptides were incubated with cell lysates from HEK293 cells expressing the G-CSFR and SOCS-3 (Fig. 4A). The peptides were precipitated with SA Sepharose and coprecipitated SOCS-3 was visualized by immunoblotting. Fig. 4A shows that SOCS-3 preferentially binds to a peptide containing pY729. Small amounts of SOCS-3 were also coprecipitated with the peptide encompassing pY704.

To test whether SOCS-3 directly binds to the G-CSFR peptides, we incubated purified rSOCS-3 with biotinylated peptides linked to SA-coupled Sepharose. Fig. 4B shows that purified SOCS-3 was readily coprecipitated with the pY729 peptide, showing direct binding to this motif. Nonphosphorylated peptides did not show any interaction with SOCS-3. The direct interaction of SOCS-3 to specific G-CSFR-peptides thus is pY-dependent and sequence-specific.

Peptide precipitation assays performed with cells overexpressing SOCS-1 showed no interaction with the peptides of the G-CSFR (Fig. 4C).

**SOCS-3 binds to pY729 within the G-CSFR**

To assess the relevance of SOCS-3 binding to the phospho-Y729 and -Y704 motifs within the G-CSFR, we generated G-CSFR mutants where either Y704 or Y729 were substituted with phenylalanine. HEK293 cells which have been transduced with SOCS-3 or a control vector were then transiently transfected with the mutated receptors. As shown in Fig. 5, SOCS-3 was coimmunoprecipitated with the G-CSFR in a stimulation-dependent manner in cells carrying the mutated receptor G-CSFR/Y704F. However, immunoprecipitation of the mutant receptor G-CSFR/Y729F failed to coprecipitate any SOCS-3 protein.

**Mutation of pY729 prevents SOCS-3-mediated inhibition of G-CSF-induced gene expression**

To determine the importance of SOCS-3 recruitment to pY729 for G-CSF-induced signal transduction, we performed reporter gene assays in HEK293 cells that were stably transfected with cDNAs encoding the G-CSFR point mutants Y704F or Y729F and a luciferase reporter construct responsive to STAT1, STAT3, and STAT5. The cells were retrovirally transduced with a construct coding either for GFP or SOCS-3. Cells were stimulated with G-CSF and luciferase activity was measured after 6 h. Reporter assays in cells carrying a Y704F mutant showed similar suppression of the luciferase signal (Fig. 6A) as observed with the wild-type receptor (shown in Fig. 2). The suppressive effect of SOCS-3 could be overridden by mutating tyrosine Y729 to phenylalanine (Fig. 6B). The control experiment with cells transfected with a vector containing GFP alone gave similar dose-response curves as the mock-transduced cells and the same maximal response was maintained.

**SOCS-3 binds to pY729 with high affinity**

Next, the affinity of the interaction between SOCS-3 and the G-CSFR was determined. Phosphorylated and nonphosphorylated G-CSFR peptides were immobilized on SA chips and the interaction with purified rSOCS-3 was measured by means of surface plasmon resonance (SPR). Fig. 7, A and C, shows the sensograms of the two phosphopeptides pY729 and pY704 which interact with SOCS-3. $K_D$ was calculated by Scatchard analysis (Fig. 7, B and D). The SPR data (Table I) revealed a calculated $K_D$ of 2.8 μM for pY729 and 6.8 μM for pY704. The phosphopeptides pY744 and pY764 revealed $K_D$ values higher than 30 μM. Nonphosphorylated peptides which served as negative controls showed no interaction with SOCS-3.

**Discussion**

The G-CSFR is a cytokine receptor responsible for the differentiation and development of hemopoietic precursor cells into the myeloid lineage, namely granulocytes and monocytes (40, 41). It has been shown that G-CSF, the natural ligand for the G-CSFR, activates the JAK/STAT, as well as the mitogen-activated protein kinase, pathway (12, 13, 16, 19, 42–44), but little is known so far about the negative regulation of the G-CSF-mediated signal transduction. It was shown that G-CSF activates the SH2-containing phosphatase-1 and -2, the serine/threonine kinase Akt (protein kinase B) as well as SH2 domain-containing inositol phosphatase-1.
eloid cell differentiation (17, 51), STAT5 seems to be involved in G-CSF-dependent cell proliferation (18). Because we found a potential induction of SOCS-3 mRNA in U937 cells as well as in primary neutrophils upon G-CSF stimulation, we examined the impact of SOCS-3 on G-CSF-induced STAT DNA binding by means of a STAT-luciferase assay. In these experiments, we observed a significant (p < 0.001) reduction in luciferase activity suggesting that SOCS-3 acts as a feedback inhibitor of G-CSF-mediated signal transduction (Fig. 2A). A dose-response analysis revealed suppression of the maximal response in SOCS-3-expressing cells by 74%. In addition, we demonstrate that SOCS-1 also negatively regulates G-CSF-mediated signal transduction (Fig. 2B).

Like SOCS-1, SOCS-3 was initially shown to inhibit JAK/STAT signaling by binding to and inhibiting JAK (22–24). We and others have recently shown that SOCS-3 not only interacts with JAK, but also exerts at least part of its inhibitory effect by binding to the phosphorylated IL-6-type cytokine receptor subunit gp130 (31, 32). Because the G-CSF shares ~45% homology with gp130 and these two receptors are structurally related to a great extent, it was important to determine whether there is a similar inhibitory mechanism mediated by SOCS-3. We performed coimmunoprecipitation experiments and could show that SOCS-3 interacts with the G-CSF in a stimulation-dependent manner (Fig. 3). Furthermore, we were able to coimmunoprecipitate endogenous G-CSF with endogenous SOCS-3 in a G-CSF-dependent manner, indicating that the interaction is of physiological relevance (Fig. 3C). This, together with other recent data (36–39, 52), strongly suggests that recruitment of SOCS-3 to cytokine receptor pY motifs is required for SOCS-3 to negatively regulate cytokine signaling.

To identify the recruitment site for SOCS-3 on the G-CSF, we performed a peptide precipitation assay with phosphorylated and nonphosphorylated biotinylated peptides comprising one of the four tyrosine motifs of the cytoplasmic part of the G-CSF. We found that pY729 was the major binding site for SOCS-3 and determined a K_D of 2.8 μM for this interaction. In addition, we found pY704 to interact with SOCS-3, the calculated K_D for this interaction being around 6.8 μM. For comparative reasons, K_D values of the SOCS-3/G-CSF peptide interaction were set into relation with dissociation constants of known interaction partners of SOCS-3. We similarly performed SPR measurements with SOCS-3 and a biotinylated gp130 peptide comprising the binding motif for SOCS-3, namely pY759. In these experiments, a K_D of ~210 nM was obtained. The determined affinity is in the same range of the affinity measured in a recent study reporting a K_D value of 42 nM for this interaction (32). This difference may be explained by the use of peptides corresponding to the amino acid sequence of human gp130 (pY759 motif) (56), amino acid sequence: βA-TSSTVQpYSTVVHSG) vs murine gp130 (pY757 motif, Ref. 32, amino acid sequence: acetyl-STAStVEpYSTV VHSG) and, in contrast, by differences in the experimental conditions used in both studies. The G-CSF peptide binds SOCS-3 with ~13-fold less avidity than the pY759 motif of gp130.

In addition, we examined the interaction of SOCS-3 with G-CSF and showed that SOCS-3 interacts with G-CSF in a stimulation-dependent manner (Fig. 4C). This is in agreement with the established inhibitory mechanism of SOCS-1 through direct binding to the kinases of the Janus family (23, 24, 53) and emphasizes the difference in the inhibitory mechanisms of SOCS-1 and SOCS-3.

The alignment of the peptide sequence around the tyrosine motifs Y729 and Y704 of the G-CSFR with known SOCS-3 recruiting motifs (Table II) (31, 32, 37–39, 52) shows a significant sequence homology in the case of the motif pY729 whereas the amino acid sequence around pY704 shows no homology with the proposed

**FIGURE 6.** Mutation of Y729 suppresses the SOCS-3-mediated inhibition of G-CSFR induced gene expression. Mock-, GFP-, and SOCS-3-transduced HEK293 cells carrying the G-CSFR mutants Y704F (A) or Y729F (B), and a STAT-luciferase response element were stimulated with G-CSF at concentrations ranging from 0.2 pg/ml to 1 μg/ml with a serial dilution factor of 4. Luciferase activity was measured 6 h after stimulation. Luciferase response at maximal G-CSF concentration was set to 100% and the percentage of stimulation at 1 μg/ml G-CSF was calculated.

which are suggested to modulate G-CSF signal transduction (20, 45, 46). SOCS proteins have been shown to inhibit cytokine signaling in a classical feedback loop mechanism (21–24). We were interested in the role of SOCS-3 in regard to G-CSF signal transduction. In a first step, we investigated whether G-CSF induces SOCS-3 mRNA in the myeloid precursor cell line U937 as well as in primary PMN. Treatment with human rG-CSF resulted in a dramatic increase of SOCS-3 mRNA within 45 min. This rapid induction of SOCS-3 was similar to that observed with other cytokines (47–49) and this indicates that SOCS-3 is an immediate early gene induced by G-CSF to rapidly modulate G-CSF-mediated signaling. The strong and rapid induction of SOCS-3 mRNA in myeloid cells by G-CSF reported in this study, together with the previously reported potent induction of SOCS-3 by G-CSF, GM-CSF, and IL-3 in bone marrow cells (22), is a strong indication for SOCS-3 being an important regulator of signal transduction in immune cells. G-CSF also rapidly induced SOCS-1 expression, in bone marrow cells (22), and needs further investigation.

STAT1, STAT3, and STAT5 have been shown to be activated by G-CSF (16, 18, 50). Although STAT3 has been linked to myeloid cell differentiation (17, 51), STAT5 seems to be involved in G-CSF-dependent cell proliferation (18). Because we found a potential induction of SOCS-3 mRNA in U937 cells as well as in primary neutrophils upon G-CSF stimulation, we examined the impact of SOCS-3 on G-CSF-induced STAT DNA binding by means of a STAT-luciferase assay. In these experiments, we observed a significant (p < 0.001) reduction in luciferase activity suggesting that SOCS-3 acts as a feedback inhibitor of G-CSF-mediated signal transduction (Fig. 2A). A dose-response analysis revealed suppression of the maximal response in SOCS-3-expressing cells by 74%. In addition, we demonstrate that SOCS-1 also negatively regulates G-CSF-mediated signal transduction (Fig. 2B).

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In comparison, SOCS-1, which we also found to be induced by G-CSF, did not interact with G-CSFR-derived phosphopeptides (Fig. 4C). This is in agreement with the established inhibitory mechanism of SOCS-1 through direct binding to the kinases of the Janus family (23, 24, 53) and emphasizes the difference in the inhibitory mechanisms of SOCS-1 and SOCS-3.

The alignment of the peptide sequence around the tyrosine motifs Y729 and Y704 of the G-CSFR with known SOCS-3 recruiting motifs (Table II) (31, 32, 37–39, 52) shows a significant sequence homology in the case of the motif pY729 whereas the amino acid sequence around pY704 shows no homology with the proposed...
consensus sequence h-X-pY-h/S-T-X-L/V-h-h (with h = hydrophobic) optimal for SOCS-3 recruitment (52). Subsequent coimmunoprecipitation experiments, as well as reporter gene assays with either wild-type G-CSFR or the Y704F and Y729F mutants, were performed to clarify the role of the two tyrosine motifs in regard to the SOCS-3 action in the context of the full-length G-CSFR and the cellular environment. We observed a complete loss of SOCS-3 binding upon mutation of Y729, strongly suggesting that pY729 is the physiological binding site for SOCS-3 and that pY704 does not play a significant role in recruiting SOCS-3 in the context of the full-length G-CSFR (Fig. 5). Reporter gene assays performed with the wild-type receptor, as well as with Y704F and Y729F receptor, mutants revealed that mutation of Y704 has no effect on the suppression of G-CSF-mediated STAT activation by SOCS-3. In contrast, substitution of Y729 to phenylalanine resulted in luciferase activities similar to those found in control or mock-transduced cells. These data, together with the data from our coimmunoprecipitation experiments, strongly suggest that Y729 of the G-CSFR is essential for the inhibitory effect of SOCS-3 on G-CSF-mediated signal transduction.

G-CSF is commonly used for the treatment of SCN (54). Approximately 10% of SCN patients develop acute myeloid leukemia (7, 8) and a correlation between the acquisition of G-CSFR mutations and the leukemic progression of the disease has been reported (9–11). These mutations introduce premature stop codons between codons 714 and 732 and a recent report provides evidence that a G-CSFR truncated at amino acid position 715 results in sustained STAT5 activation and a hyperproliferative response in mice carrying this mutation (55). Although a slower internalization rate of the mutated G-CSFR was found in these mice and could be responsible for this response, we suggest that the absence of the

Table I. Calculated $K_D$ values for the interaction between G-CSFR peptides and SOCS-3 as determined by Scatchard analysis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pY704</td>
<td>6.8 ± 0.0069</td>
</tr>
<tr>
<td>pY729</td>
<td>2.8 ± 0.0162</td>
</tr>
<tr>
<td>pY744</td>
<td>&gt;30</td>
</tr>
<tr>
<td>pY764</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

Table II. Sequence comparison of pY729 and pY704 with receptor pY motifs known to recruit SOCS-3

<table>
<thead>
<tr>
<th>Receptor</th>
<th>pY Location</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-gp130</td>
<td>759 STVQ</td>
<td>pYS T V H</td>
<td>31, 32</td>
</tr>
<tr>
<td>m-Leptin</td>
<td>985 PSVK</td>
<td>pY A L V S</td>
<td>38, 39</td>
</tr>
<tr>
<td>m-Leptin</td>
<td>1077 KSV C</td>
<td>pY L G T S</td>
<td>38</td>
</tr>
<tr>
<td>h-Epo</td>
<td>401 ASFE</td>
<td>pY T L D</td>
<td>37</td>
</tr>
<tr>
<td>h-Epo</td>
<td>429 PHL K</td>
<td>pY L V V</td>
<td>52</td>
</tr>
<tr>
<td>h-G-CSF</td>
<td>729 DQVL</td>
<td>pY G Q L G</td>
<td>38</td>
</tr>
<tr>
<td>h-G-CSF</td>
<td>704 LVTQ</td>
<td>pY V L Q G D</td>
<td></td>
</tr>
</tbody>
</table>

* Bold characters highlight conserved residues.

h, Human; m, murine.
SOCS-3 recruiting motif pY729 identified in the present study also contributes to this increased and prolonged STAT activation. Taken together, we have shown that SOCS-3 is strongly and rapidly induced by G-CSF in myeloid cells and in turn potently inhibits G-CSF-mediated signal transduction. We provide evidence that the inhibitory action of SOCS-3 on G-CSF signaling involves the direct binding of SOCS-3 to the activated G-CSFR and we identify pY729 as being the recruitment site for SOCS-3. To evaluate the implications of SOCS-3 recruitment to the receptor motif pY729 on the various signaling components involved in G-CSF signal transduction, further studies are necessary and are currently under investigation.

Acknowledgments

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References


