Oncostatin M Receptor-mediated Signal Transduction Is Negatively Regulated by SOCS3 through a Receptor Tyrosine-independent Mechanism*

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Down-regulation of interleukin (IL)-6-type cytokine signaling has been shown to occur, among other mechanisms, via induction of the feedback inhibitor SOCS3 (suppressor of cytokine signaling 3). Binding of SOCS3 to the phosphorylated Tyr759 in the cytoplasmic region of gp130, the common signal transducing receptor chain of all IL-6-type cytokines, is necessary for inhibition of Janus kinase-mediated signaling. In the present study, we analyzed the effect of SOCS3 on signal transduction by the proinflammatory cytokine oncostatin M (OSM), which signals through a receptor complex of gp130 and the OSM receptor (OSMR). OSM leads to a much stronger and prolonged induction of SOCS3 in HepG2 hepatoma cells and murine embryonal fibroblasts (MEF) compared with IL-6. A negative effect of SOCS3 on OSM signaling was confirmed using MEF cells lacking SOCS3. We can show that the OSMR-mediated signaling is inhibited by SOCS3 to a similar extent as previously described for gp130. However, the inhibition occurs independent of tyrosine motifs within the OSMR. Instead, SOCS3 interacts directly with JAK1 in a stimulation-dependent manner, a mechanism so far only known for SOCS1.

The 28-kDa protein oncostatin M (OSM)3 belongs to the family of interleukin-6-type cytokines, which comprises the largest known cytokine family with to date nine members. Many of the family members, particularly interleukin-6, are known to regulate inflammatory processes, at the forefront the acute phase response (1). Their elevated expression levels in many pathophysiological situations like coronary heart disease (2), inflammatory bowel disease, Castleman disease, rheumatoid arthritis (3), or psoriasis (4) have led many researchers to design promising strategies to inhibit the action of these cytokines.

All IL-6-type cytokines signal via receptor complexes containing at least one gp130 (glycoprotein 130) (5). Depending on the second participating signal transducing receptor chain the family can be further subdivided. IL-6 and IL-11 use a homodimer of two chains of gp130; leukemia inhibitory factor, cardiotoxin-1, cardiotoxin-like cytokine, ciliary neurotrophic factor, neuroepoetin, and OSM signal via a heterodimer of gp130 and the leukemia-inhibitory factor receptor; OSM is the only family member that additionally signals via a gp130-OSM receptor (OSMR) complex; and IL-27 signals via a gp130-WSX-1 receptor complex (6, 7).

Whereas IL-6 represents one of the best studied cytokines to date, the importance and physiological activities of oncostatin M are less well known. OSM is predominantly secreted by activated T lymphocytes, macrophages, and neutrophils (8, 9) and seems to have rather proinflammatory properties (10, 11). Increased levels of this cytokine were found in the synovial fluid of rheumatoid arthritis patients (12) and in dermal lesions of psoriasis patients (4). OSMR knock-out mice display defects in hematopoiesis and liver regeneration (13, 14). It is well appreciated that OSM, besides activating the MAPK and phosphatidylinositol 3-kinase pathway, is one of the strongest inducers of the JAK/STAT pathway (6). Upon ligand binding and receptor dimerization, the Janus kinase family members JAK1, JAK2, and Tyk2 are activated and in turn mediate phosphorylation of tyrosine residues in the cytoplasmic region of either gp130 or the OSMR. These then serve as docking sites for SH2 domain-containing molecules, such as STAT factors, which after binding and phosphorylation transduce signals into the nucleus.

To avoid overstepping cytokine signaling with possible destructive effects, inhibitory mechanisms restrict the duration as well as the intensity of the activated signal transduction pathways. Besides the SH2 domain-containing phosphatase SHP-2, the feedback inhibitor SOCS3 (suppressor of cytokine signaling 3) has been described to be involved in termination of IL-6- and leukemia inhibitory factor-mediated STAT activation via binding to the phosphorylated receptor tyrosine motifs surrounding Tyr(P)759 in gp130 and Tyr(P)974 in the leukemia-inhibitory factor receptor (15–17) (reviewed in Ref. 18).

SOCS3 belongs to a family comprising eight proteins, which share two structural motifs: a central SH2-domain recognizing phosphoryrosine-containing motifs and a C-terminal SOCS box, which interacts with ubiquitin ligases to mediate proteasomal degradation of SOCS-associated substrates (19). Similar to what has been described for SOCS1 (20), the inhibitory mechanism of SOCS3 also involves binding of its SH2 domain to the phosphorylated activation loop within the JAK kinase domain. The tyrosine kinase activity seems to be inhibited via the kinase-inhibitory region (KIR) of SOCS3, which is located N-terminal to the SH2 domain (21). However, compared with SOCS1, the binding affinity of SOCS3 for the JAK phosphotyrosine motif is weaker, and it is thought that SOCS3 is recruited to the JAKs via prior binding to phosphorylated receptor motifs (15, 22).
A role for SOCS3 in inhibiting OSM signaling has been suggested by investigators before (23, 24). However, all of the studies performed so far have analyzed the wild-type OSM receptor complex, which per se contains gp130. Therefore, it was not possible to distinguish whether the inhibitory activity is based exclusively on tyrosine 759 in the gp130 part or whether the OSMR itself contains SOCS3-responsive sites too. Answering these questions has gained increasing importance, since the OSMR has recently been shown to represent the second signaling receptor subunit not only for OSM but also for the novel cytokine interleukin-31 (25). Although this cytokine is closely related to the IL-6-type cytokines, it does not signal via gp130. Instead, it uses the gp130-related receptor (GLMR, GPL, IL-31R) described independently by different groups (25–27).

Here we show that SOCS3 inhibits OSMR-mediated signaling to a similar extent as gp130-mediated signaling. Our studies in human HepG2 hepatoma cells and in murine embryonal fibroblasts demonstrate that SOCS3 is much more strongly induced by OSM than by IL-6. Interestingly, no binding site for SOCS3 in the cytoplasmic region of the OSMR could be identified. Instead, we observed enhanced binding of SOCS3 to JAK1 in response to OSM stimulation compared with IL-6 treatment. Thus, our results demonstrate that a direct binding of SOCS3 to JAK1 is possible under physiological conditions and may suggest a higher affinity of SOCS3 to the OSMR-associated JAKs compared with the gp130-associated JAKs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Growth Factors, and Transient Transfection**—Human embryonic kidney cells, SV40-transformed cells (HEK293T), and murine embryonic fibroblasts (MEF) were maintained in Dulbecco’s modified Eagle’s medium; human hepatoma cells (HepG2) were maintained in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 60 mg/ml penicillin. Cells were grown at 37 °C in a water-saturated atmosphere containing 5% CO2. SOCS3-deficient cells were described as described previously (28, 29). Recombinant human IL-5 and human OSM were obtained from R&D Systems (Minneapolis, MN). Recombinant mouse IL-5 and human OSM were obtained from Cell Concepts (Umkirch, Germany). Murine OSM was obtained from R&D Systems (Minneapolis, MN). Recombinant IL-6 and soluble human IL-6 receptor (sIL-6R) were prepared as described previously (30). HepG2 cells were transfected with 8–17 μg of vector DNA using the calcium phosphate method as described (30). HEK293T cells were transfected with FuGene6 (Roche Applied Science) according to the manufacturer’s recommendation using a ratio of 2:1.

**Expression Vectors**—The construction of the pRCMV-based expression vectors for the chimeras IL-5Ra/gp130Y759F and IL-5Rβ/gp130Y759F (31) and the pCAGGS-based expression vectors for the chimeras IL-5Ra/gp130Y759F and IL-5Rβ/OSMR have been described previously (30). For transfection of HepG2 cells, XhoI/BamH1 fragments comprising the cDNA encoding the receptor constructs IL-5Ra/gp130, IL-5Rβ/gp130, IL-5Rβ/OSMR3, and IL-5Rβ/OSMRΔ4 (30, 32) (Fig. 3A) were inserted into XhoI/BglII-digested pCAGGS expression vector. One further C-terminal deletion mutant of the OSMR, IL-5Rβ/OSMRΔ6 (Fig. 3A), was generated by polymerase chain reaction using an antisense oligonucleotide incorporating an in-frame termination codon after amino acid Asn836 followed by the recognition site for BamH1. The resulting product was cloned into the EcoRI/BamH1-digested expression plasmid pSVL-IL-5Rβ/gp130 and subcloned into the pCAGGS expression vector. Construction of pCAGGS-IL-5Rβ/OSMRβ837F was carried out by standard cloning procedures using an oligonucleotide incorporating the respectively mutated codon for the exchange from tyrosine to phenylalanine and the IL-5Rβ/OSMRwt as a template. The integrity of all constructs was verified by DNA sequence analyses using an ABI PRISM 310 Genetic Analyzer (PerkinElmer Life Sciences). The expression vectors for murine SOCS1, SOCS2, SOCS3, and CIS were pEF-FLAG-mSOCS1, pEF-FLAG-mSOCS2, pEF-FLAG-mSOCS3, and pEF-FLAG-mCIS (33), kindly provided by D. Hilton (Walter and Eliza Hall Institute, Melbourne, Australia).

**Cell Lysis, Immunoprecipitations, and Western Blotting**—HepG2 cells were stimulated with 5 and 20 ng/ml OSM, respectively, or 20 and 100 ng/ml IL-6, respectively, for the indicated times. MEF cells were stimulated with 20 ng/ml OSM or 20 ng/ml IL-6 and 0.5 μg/ml sIL-6R for the times indicated. A 40-min preincubation with the MEK1 inhibitor U0126 (Promega, Madison, WI) was used as indicated to ERK1/2 activation. Immediately after stimulation, cells were lysed in Triton X-100 lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonlfyl fluoride, 5 μg/ml aproatin, and 5 μg/ml leupeptin), as described previously (30). For coimmunoprecipitation experiments, cells were preincubated at a concentration of 10 μM of the proteasome inhibitor MG132 (Calbiochem, Merck AG, Darmstadt, Germany) for 30 min before stimulation and lysed in Brij97 lysis buffer (as Triton lysis buffer, but with 1% Brij97). Coimmunoprecipitation studies were performed as described (30). Proteins were separated by SDS-PAGE in 7.5 or 12% gels, followed by electroblotting onto a polyvinylidene difluoride membrane (PALL, Dreieich, Germany). Western blot analysis was conducted using the indicated antibodies and the enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s instructions. For reprobing, the blots were stripped in 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.7) for 25 min at 75 °C.

**Antibodies**—The monoclonal antibody against STAT3 was obtained from BD Biosciences. The phosphospecific polyclonal antisera against STAT3 (Tyr705) and p44/42 MAPK (Thr202/Tyr204) were from Cell Signaling Technology (Beverly, MA). The polyclonal antibodies against SOCS3 (M-20, p38 (C-20), and JAK1 (HR-785) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The phosphospecific p38 (Thr180/Tyr182) polyclonal antisera was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The phosphospecific p38 (Thr180/Tyr182) antisera was obtained from Promega (Madison, WI). Tyrosine-phosphorylated proteins were detected using a mixture of 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) and PY99 (Santa Cruz Biotechnology). For immunoprecipitations of SOCS3, the rabbit polyclonal antisera FAI017 (Fusion Antibodies, Belfast, UK) was used. The horseradish peroxidase-conjugated secondary antibodies were purchased from DAKO (Hamburg, Germany).

**Reporter Gene Assay**—α2M(-215)-Luc contains the promoter region −215 to +8 of the rat α2M-macroaglobulin gene upstream of the firefly luciferase-encoding sequence of plasmid pGL3 basic (Promega, Madison, WI). The IRF-1-tk-Luc construct contains the STAT1-responsive element of the IRF-1 promoter upstream of a thymidine kinase minimal promoter cloned into the pGL3 vector.

HepG2 cells were grown on 6-well plates to 30% confluence and transfected in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Calcium phosphate precipitation was performed with the indicated amounts of expression plasmids for the receptor constructs and SOCS1, -2, or -3, respectively, 2 μg of the β-galactosidase control plasmid, and 6 μg of reporter gene expression vector. Twenty-four hours after transfection, cells were stimulated with 10 ng/ml recombinant human IL-5 (Cell Concepts, Umkirch, Germany) for 16 h. Cell lysis and luciferase assays were performed using the Promega luciferase assay system (Promega, Madison, WI). All experiments were performed at least in triplicate. Luciferase activity values were...
RESULTS

OSM Strongly Induces Expression of Several SOCS Family Members in Human Hepatoma Cells as Well as in Murine Fibroblasts—The genes encoding SOCS proteins have been shown to be induced by many different cytokines, including IL-6-type cytokines, and once translated, these proteins act in a negative feedback loop to inhibit the signal transduction of these cytokines (33–35). Since the liver is one of the major target organs of OSM, highlighted by the fact that OSMR-deficient mice suffer from a defective liver regeneration (14), we first examined SOCS mRNA expression induced by OSM in the human hepatoma cell line HepG2. RNAse protection assays were performed with RNA isolated from cells stimulated for up to 2 h with OSM (Fig. 1A). We observed a very strong elevation of SOCS3 mRNA levels, which was induced as early as 15 min after stimulation with OSM with a peak at 60 min and a continuing decline up to 120 min. Additionally, mRNA levels of CIS, SOCS1, and SOCS2 were increased, however to a lower extent. SOCS5, SOCS6, and SOCS7 mRNAs were constitutively expressed and not further induced by stimulation with OSM.

OSM Is a More Potent Inducer of SOCS3 than IL-6—Since SOCS3 was the most prominently OSM-induced SOCS family member, we concentrated for the remaining studies on this feedback inhibitor and the elucidation of its molecular actions. In a next step, we therefore examined SOCS3 protein induction in HepG2 cells (Fig. 1B). Since SOCS3 has also been shown to be one of the major SOCS proteins induced by IL-6 (33), we compared the OSM- and IL-6-stimulated SOCS3 expression. Fig. 1B shows that at equal concentrations of these two cytokines, the OSM-mediated induction of SOCS3 is significantly stronger than the one mediated by IL-6, particularly at the later time point analyzed (60–120 min). Even if the amount of OSM is reduced to one-twentieth of the concentration of IL-6, no comparable amounts of...
SOCS3 protein were produced in response to IL-6, which further demonstrates the much stronger potential of OSM to induce SOCS3 (Fig. 1C). It must be noted here that we had to preincubate the cells with the proteasomal inhibitor MG132 for this experiment to stabilize the low levels of SOCS3 protein. As a result, SOCS3 protein levels were still rising after 120 min of OSM treatment and still clearly detectable in response to IL-6.

In the human system, OSM has the exceptional property of signaling via two receptor complexes, the gp130-OSMR complex and the gp130-leukemia-inhibitory factor receptor complex (36). Both types of receptor complexes could account for the strong induction of SOCS3. In order to determine the contribution of the specific OSMR to the strong induction of SOCS3, we switched to the murine system, in which OSM signals only via the gp130-OSMR complex (37). Time kinetic studies were performed in murine embryonic fibroblasts (MEFs) stimulated with murine OSM or human IL-6 in combination with its soluble IL-6R, respectively (Fig. 1D). It has to be noted here that the human IL-6/sIL-6R complex binds and activates the murine gp130 with the same affinity as human gp130 (38). As seen before in HepG2 cells, also in MEF cells, OSM led to a strong induction of SOCS3 after 30 min and was still increasing at 90 min of stimulation. The IL-6-induced SOCS3 expression was much weaker and returned to basal levels already after 60 min of treatment. Compared with HepG2 cells, MEFs responded to cytokine stimulation with a much earlier robust SOCS3 induction. Whereas in HepG2 at 30 min only marginal SOCS3 levels could be detected (Fig. 1, B and C), the MEFs showed already an almost full response (Fig. 1D).

Taken together, these results strongly suggest that the strong up-regulation of SOCS3 by OSM is mediated by the gp130-OSMR complex. Interestingly, in both cell types, the OSM-induced SOCS3 expression is prolonged compared with IL-6.

**SOCS3 Production Is Enhanced through an MEK1/2-dependent Pathway**—SOCS3 expression has been shown to be critically dependent on STAT1 and/or STAT3 (35, 39, 40). Additionally, there have been several reports suggesting a role for members of the MAPK family in induction of SOCS3 (20, 41–43). Whereas OSM and IL-6 activate STAT3 to a comparable extent, we showed recently that OSM has a much higher potential to induce phosphorylation of the MAPK family members ERK1/2 as well as the stress-activated MAPKs, p38 and c-Jun N-terminal kinase (44). Therefore, we analyzed the role of MAPKs in OSM-mediated expression of SOCS3 (Fig. 2A). HepG2 cells were pretreated for 40 min with the MEK1/2 inhibitor U0126 and stimulated with OSM for the times indicated. OSM-induced SOCS3 expression after 60 min of treatment is strongly reduced by preincubation of the cells with U0126 (Fig. 2A, *first panel*), which abolishes ERK1/2 phosphorylation (Fig. 2A, *fourth panel*). STAT3 phosphorylation, on the other hand, is prolonged by pretreatment with the MEK inhibitor (*second panel*). This led us to the conclusion that ERK1/2 MAPKs contribute substantially to the strong OSM-mediated induction of SOCS3 observed in HepG2 cells.

**SOCS3 Counteracts OSM-mediated Signal Transduction**—It has been described that SOCS3 induced by IL-6 acts in a negative feedback loop to down-regulate IL-6 signaling (33, 45). To see if this holds true also for OSM-induced signal transduction, we took advantage of MEF cells in which the SOCS3 gene has been deleted (28, 29). These cells and wild-type cells were incubated for the indicated times with OSM, and whole cellular extracts were analyzed for the activation of JAK1, STAT3, and ERK1/2. Compared with wild-type cells, SOCS3-lacking cells display an enhanced and prolonged activation of JAK1, STAT3, and ERK1/2 in response to OSM stimulation (Fig. 2B). STAT3 and ERK1/2 phosphorylation in wild-type cells exhibit a biphasic activation profile with a maximum at 15 min, a decrease after 60 min, and a slight recovery after 90–120 min, which inversely correlates with SOCS3 protein levels maximal at 60 min and decreasing again after 90 min. In cells lacking SOCS3, however, the initial induction of JAK1, STAT3, and ERK1/2 activation is stronger, and no time-dependent reduction in phosphorylation levels can be detected up to 120 min poststimulation. Due to the lack of suitable antibodies to immunoprecipitate the murine OSM receptor, we performed co-immunoprecipitation studies using an antibody against JAK1. Indeed, as seen in whole cellular extracts, JAK1 tyrosine-phosphorylation is prolonged in SOCS3-deficient cells (Fig. 2B, *upper panel, right*). A tyrosine-phosphorylated protein of ~180 kDa is co-precipitated with JAK1. Since the molecular mass of the OSMR is 180 kDa, we assume that this protein is the OSMR, but there are no antibodies to detect the protein in Western blots. Taken together, these observations indicate a role for SOCS3 as a negative feedback inhibitor for OSM signal transduction.

**The Inhibitory Effect of SOCS3 on the OSMR Complex Is Comparable with the Effect on the IL-6R Complex**—SOCS3 has been shown to be recruited to the phosphorylated tyrosine residue 759 within the cytoplasmic part of gp130 (15), thereby inhibiting gp130-dependent signal transduction. So far, no binding site for SOCS3 within the OSMR has been described. In order to determine whether the negative regulatory potential of SOCS3 on a gp130/gp130 homodimeric receptor complex (as activated by IL-6) is comparable with the one on a gp130-OSMR heterodimeric receptor complex (as activated by OSM), we utilized a chimeric receptor system established in our laboratory before (30–32). These receptor constructs consist of the IL-5R α- and β-extracellular parts fused to the transmembrane and intracellular parts of gp130 or the OSMR, respectively (Fig. 3A), and allow the analysis of mutated cytokine receptors independent of endogenously expressed receptors.

Increasing amounts of SOCS3 cDNA (10–2000 ng) were cotransfected with expression vectors for chimeric receptors mimicking either the IL-6R complex (IL-5Ra/gp130 and IL-5Rβ/gp130) or the OSMR complex (IL-5Ra/gp130 and IL-5Rβ/OSMR) and a STAT3-responsive α2-macroglobulin promoter-driven reporter gene. Interestingly, the stimulation-dependent induction of the reporter gene expression was significantly reduced by coexpression of 100 ng of SOCS3 cDNA in both cases (Fig. 3B).

To focus on the OSM-initiated signaling, we compared the inhibition of reporter gene induction via the two receptor complexes IL-5Ra/gp130YFFFFF + IL-5Rβ/gp130 and IL-5Ra/gp130YFFFFF + IL-5Rβ/OSMR, respectively (Fig. 3C). In each of these receptor complexes, the tyrosine-based signal transduction of one gp130 chain has been abrogated. Since these mutated tyrosines include tyrosine 759, we additionally prevented SOCS3 recruitment to the mutated gp130 chain. Nevertheless, the inhibitory effect of SOCS3 on signaling is not only clearly detectable if one gp130-chain remains intact (Fig. 3C, *left*), but also if only the OSMR contains intact tyrosine motifs (Fig. 3C, *right*). Indeed, SOCS3 seems to be more effective on the OSMR than on gp130, since cotransfection of only 10 ng of cdNA was sufficient to achieve half-maximal inhibition of OSMR-mediated reporter gene expression. These data point out that also the OSMR itself contains SOCS3-susceptible regions in its intracellular tail.

OSM does not exclusively induce the enhanced expression of SOCS3 but additionally induces SOCS1, SOCS2, and CIS (Fig. 1). Thus, we investigated if all of these four proteins inhibit OSM-mediated signal transduction. HepG2 cells were cotransfected with cdNAs for the SOCS family members CIS, SOCS1, SOCS2, and SOCS3, the chimeric receptors indicated, as well as luciferase reporter genes with promoters responsive for STAT3 (Fig. 4A) or STAT1 (Fig. 4B). Reporter gene induction in response to stimulation of the chimeric receptor com-
plexes with IL-5 was examined. Overexpression of SOCS1 and SOCS3 reduces the induction of the STAT3-responsive α2M-promoter-reporter gene construct (Fig. 4A) as well as the induction of the STAT1-responsive IRF-1 promoter-reporter gene construct (Fig. 4B). In contrast to SOCS3, SOCS1 has been shown to inhibit JAK/STAT signaling by directly binding to the JAK activation loop, thereby inhibiting JAK kinase activity and the resulting reporter gene activation (20).

The OSMR Does Not Contain a Binding Site for SOCS3—The results obtained so far pointed to a direct binding of SOCS3 to the OSMR similar to gp130. Comparison of the sequences of the OSMR tyrosine motifs with the consensus sequence for SOCS3 recruitment (46) and already described recruitment sites at other receptors (15, 47–50) revealed a possible interaction motif: YLYLLP. Therefore, reporter gene assays as described for Fig. 3C were performed with a receptor complex in which Tyr837 within IL-5Rα/OSMR was mutated to phenylalanine (IL-5Rα/gp130YFFFFF and IL-5Rβ/OSMR Y837F) (Fig. 5A).

To our surprise, reporter gene induction via this receptor complex is inhibited by SOCS3 comparably with the IL-5Rα/gp130YFFFFF + IL-5Rβ/OSMRwt receptor complex (Fig. 3C, right). In order to analyze whether any other region within the cytoplasmic part of the OSMR is responsible for an interaction with SOCS3, we made use of deletion mutants of the OSMR cytoplasmic part. HepG2 cells were transfected to express SOCS3 together with the different receptor complexes indicated in Fig. 5B. The amount of SOCS3 cDNA transfected (100 ng) does not inhibit signal transduction via a gp130-receptor complex lacking both SOCS3 recruitment sites (IL-5Rα/gp130Y759F and IL-5Rβ/gp130Y759F) (Fig. 5B, compare first and second pairs of columns). This clearly indicates that no artificial direct binding of overexpressed SOCS3 to the gp130-associated JAKs occurs in this experimental setup. On the contrary, reporter gene induction in response to activation of a receptor complex composed of IL-5Rα/gp130Y759F and IL-5Rβ/OSMR is inhibited by cotransfection of SOCS3 (compare third and fourth pairs of columns), confirming our previous findings that the SOCS3 recruitment site in gp130 is not absolutely essential for down-regulation of
FIGURE 3. Gp130- and OSMR-mediated induction of the \( \alpha \)_M-promoter driven reporter gene displays a comparable sensitivity toward SOCS3. A, schematic representation of the chimeric receptors used in this study. The extracellular domains of the IL-5R\( \alpha \) or IL-5R\( \beta \) were fused to the indicated transmembrane and cytoplasmic receptor regions. Tyrosine residues in the intracellular regions are indicated as lines. The box 1 and box 2 regions are depicted as hatched boxes. B, HepG2 cells were transiently transfected with expression vectors encoding the chimeric receptors \( \alpha \)/gp130 and \( \beta \)/gp130 (20 ng each) or \( \alpha \)/gp130 (20 ng) and \( \beta \)/OSMR (80 ng) along with an expression vector for the \( \alpha \)M(1-215)-Luc reporter gene and the indicated amounts of SOCS3 cDNA. One day after transfection, cells were stimulated with 10 ng/ml IL-5 for 16 h (gray columns) or left untreated (white columns). Luciferase activities of lysates were normalized to the activity of coexpressed \( \beta \)-galactosidase. Representative activities of three independent experiments carried out in triplicates (mean \pm S.D.) are depicted. C, HepG2 cells were transiently transfected with expression vectors encoding the chimeric receptors \( \alpha \)/gp130YFFFFF and \( \beta \)/gp130 (20 ng each) or \( \alpha \)/gp130YFFFFF (20 ng) and \( \beta \)/OSMR (80 ng) along with an expression vector for the \( \alpha \)M(1-215)-Luc reporter gene and the indicated amounts of SOCS3 cDNA. Stimulation, lysis, and analysis were carried out as described in B. Representative activities of three independent experiments carried out in triplicates (mean \pm S.D.) are depicted.
OSM-induced signaling. However, activation of the αM-promoter-luciferase reporter gene via the receptor complexes containing cytoplasmic deletions of the OSMR (IL-5Ra/gp130Y759F and IL-5Rβ/OSMRΔ3, IL-5Rβ/OSMRΔ4 and IL-5Rβ/OSMRΔ6) can still be inhibited by SOCS3 (fifth to tenth pair of columns). These data show that the box 1/2 region within the OSMR chain, necessary for the JAK-receptor interaction, is sufficient to mediate the inhibitory activity of SOCS3 on OSM signaling.

To further confirm our observations, we performed peptide precipitations with peptides encompassing OSMR tyrosine motifs to precipitate SOCS3 expressed in HEK293T cells. We used peptides containing all tyrosine motifs C-terminal to the box 1/2 region in the intracellular region of the OSMR except Tyr978, which is the second last amino acid in the OSMR and therefore unable to recruit SH2 domain-containing proteins. Fig. 5C shows that in contrast to the peptide containing the described SOCS3 recruitment motif of gp130 (gp130 Tyr(P)759) (15) none of the tested phosphotyrosine-containing peptides deduced from the OSMR sequence is able to interact with SOCS3.

SOCS3 Interacts with JAK1 in Response to OSM Stimulation—Our previous results show that SOCS3 is induced by OSM and negatively regulates OSM-mediated signaling. Nevertheless, no binding site for SOCS3 in the OSMR could be identified. Therefore, we proposed a direct binding of SOCS3 to the OSMR-associated JAKs. To investigate this issue in more detail, we performed coprecipitation experiments with lysates of OSM- or IL-6-treated HepG2 cells (Fig. 6, A and B). The cells were incubated with the proteasome inhibitor MG132 prior to stimulation to stabilize the expressed SOCS3 protein. We were able to coprecipitate JAK1 together with SOCS3 after treatment of the cells with OSM for 30–90 min. It should be noted that even small amounts of SOCS3 expressed after 30 min, undetectable in whole cellular extracts (Fig. 6A, second panel), were sufficient to interact with JAK1. In contrast, no clear interaction of SOCS3 with JAK1 was found after treatment with IL-6 (Fig. 6A).

However, as shown in previous experiments, the induction of SOCS3 by OSM is much stronger compared with IL-6 (see also Fig. 1). Therefore, we treated the cells with a lower concentration of OSM to induce less SOCS3 (Fig. 6B). In this case, STAT3 activation is comparable between OSM and IL-6 (third panel). Nevertheless, the SOCS3 induction is still stronger after OSM stimulation, and again, the coprecipitation of JAK1 with SOCS3 is only clearly visible for OSM-stimulated cells. To our knowledge, these are the first results demonstrating an association of JAK1 and SOCS3 in vivo.

DISCUSSION

SOCS3 has been described to be an important negative regulator of IL-6-type cytokine signaling (33). A deletion of SOCS3 by gene targeting results in embryonic lethality due to placental defects caused by dysregulated leukemia inhibitory factor signaling (51). Studies with mice in which the SOCS3 gene has been specifically deleted in the liver or in macrophages have revealed a key role for SOCS3 in the regulation of IL-6 signaling, leading to enhanced STAT1 and STAT3 activation and an increased antiproliferative effect in macrophages (45). The mechanism by which SOCS3 exerts its inhibitory effect on IL-6 signaling has been well studied. A binding site for SOCS3 at the common signal transducing receptor of all IL-6-type cytokines, gp130, has been described (15). In contrast to down-regulation of IL-6-mediated signaling by SOCS3, less is known about the role of this feedback inhibitor in OSM signal transduction.

In this study, we could show the following: 1) OSM is a very potent inducer of SOCS3 in HepG2 and in MEF cells, and SOCS3 acts in a negative feedback loop to down-regulate OSM signaling; 2) the molecular mechanism of inhibition does not only involve binding of SOCS3 to the Tyr(P)759 motif in the gp130 chain of the OSMR complex, but additionally signaling through the OSMR itself can be inhibited by SOCS3; 3) in contrast to the inhibitory mechanisms of SOCS3 described so far,
no binding of SOCS3 to the OSMR was found, but a direct association with JAK1 was found.

Analyzing the expression of SOCS family members in response to OSM, we found the most prominent induction for SOCS3 and a weaker induction of CIS, SOCS1, and SOCS2 (Fig. 1). We could confirm that SOCS3 induced by OSM is acting in a negative feedback loop, since OSM-mediated signaling is enhanced and prolonged in the absence of SOCS3 (Fig. 2A). SOCS3 expression in response to OSM has also been described in other cell types (23, 24, 52); additionally, CIS and SOCS1 induction was found in A375 melanoma cells (23). When we compared the SOCS3 protein expression in response to OSM with the well established induction by IL-6, we noticed that OSM has a much higher potential to induce the expression of this feedback inhibitor. Differences in the signal transduction pathways activated by these two cytokines could explain this observation. So far, a critical role of the STAT transcription factors in the induction of SOCS3 gene expression is well established. STAT binding elements have been described in the murine as well as the human

FIGURE 5. The inhibitory effect of SOCS3 on OSMR-mediated signaling is independent of receptor tyrosine motifs. A, HepG2 cells were transiently transfected with expression vectors encoding the chimeric receptors α/gp130YFFFFF (20 ng) and β/OSMRY837F (80 ng) along with the indicated amounts of an expression vector for SOCS3 as well as the α/MI(–215):Luc reporter gene. One day after transfection, cells were stimulated with 10 ng/ml IL-5 for 16 h (gray columns) or left untreated (white columns). Luciferase activity of lysates was normalized to the activity of coexpressed β-galactosidase. Representative activities of three independent experiments carried out in triplicates (mean ± S.D.) are depicted. B, HepG2 cells expressing the chimeric receptors α/gp130Y759F (20 ng of cDNA) together with either β/gp130Y759F (20 ng), β/OSMR or the indicated deletion mutants of the OSMR (80 ng each), and SOCS3 (100 ng cDNA) along with the α/MI(–215):Luc reporter gene were stimulated with 10 ng/ml IL-5 for 16 h (gray columns) or left untreated (white columns). Luciferase activity of lysates was normalized to the activity of coexpressed β-galactosidase. Representative activities of three independent experiments carried out in triplicates (mean ± S.D.) are depicted. C, HEK293T cells were transfected with an expression vector encoding FLAG-tagged SOCS3 (2 μg). Lysates were prepared and subjected to precipitations with biotinylated peptides encompassing the amino acid sequence surrounding the indicated tyrosine residues of the OSMR and gp130, respectively. Peptides were precipitated using NeutrAvidin-coupled agarose, complexes were separated by SDS-PAGE, and Western blots were probed with a polyclonal antibody against SOCS3.
SOCS3 promoters (35, 39, 40). Certainly, all IL-6-type cytokines strongly activate the JAK-STAT pathway, particularly STAT3 (6). Additionally, activation of the ERK1/2 MAPK cascade has been described, which is, however, much more efficiently induced by OSM than by IL-6 (30). Furthermore, we recently described the exceptional capability of OSM to activate MAPK of all three families (i.e. ERK1/2, p38, and c-Jun N-terminal kinase) (44). Therefore, we were interested to investigate whether the strong induction of SOCS3 by OSM is dependent on the activation of these pathways. We demonstrate that inhibition of MEK1/2 reduces the expression of SOCS3, suggesting that the downstream effector kinases, ERK1/2, contribute to the strong up-regulation of SOCS3 in response to OSM (Fig. 2A). This reduced SOCS3 expression results in a prolonged STAT3 tyrosine phosphorylation (Fig. 2A, second panel), indicating that the SOCS3 expression level directly correlates with the duration of STAT3 activation.

Indeed, several studies implicate ERK1/2 or p38 in SOCS3 expression. For example, Terstegen et al. (43) observed SOCS3 expression in response to phorbol 12-myristate 13-acetate, which is inhibited by preincubation with the MEK1/2 inhibitor PD98059. In another report, Dalpke et al. (42) found an ERK1/2- and to a lesser extent a p38-dependent induction of SOCS3 by CpG-DNA. A more prominent role for p38 was suggested for IL-6-induced SOCS3 expression (41). We also analyzed the effect of the p38 inhibitor SB202190 on OSM-mediated SOCS3 expression; however, we could not verify a reproducible negative effect of this inhibitor (data not shown). Therefore, our data suggest that ERK1/2 MAPKs could act together with the STAT transcription factors to induce the high expression of SOCS3 in response to OSM.

The specific OSMR complex is composed of gp130 and the OSMR, and it is well established that SOCS3 binds to Tyr(P)759 of gp130, thereby inhibiting signal transduction (15, 22). Therefore, we were interested to see whether the inhibitory effect of SOCS3 on OSM signaling is mediated via binding to gp130 alone or whether SOCS3 also interacts with the OSMR. In reporter gene assays, overexpressed SOCS3 down-regulated signaling mediated by an OSMR complex in which gp130 signal transduction and SOCS3 binding is prevented (IL-5Rα/gp130YFFFFF and IL-5Rβ/OSMR). Indeed, at low levels of SOCS3 expression, inhibition of the OSMR-mediated signaling comparable with that of gp130 was observed (Fig. 3C). In contrast to direct binding of SOCS3 to JAKs, the inhibitory mechanism of SOCS3 is known to involve receptor binding as mentioned before. Therefore, our results suggested an interaction of SOCS3 with the OSMR similar to the recruitment to gp130. Recently, the erythropoietin receptor (EpoR) sequence surrounding Tyr(P)429/Tyr(P)431 (LKpYlPylLVVS) has been described to interact with SOCS3 with high affinity (47). In the same study, the most important residues involved in specific binding to the SOCS3 SH2 domain, based on a model structure, were suggested to be the −2, +1, and +3-positions relative to the phosphorylated tyrosine residue. The OSMR contains a tyrosine motif (Tyr837/Tyr839) very similar to the described EpoR motif, PNYLYLLPT, in which the amino acids in positions −2, +1, and +3 also have hydrophobic side chains. It also fits quite well to the described SOCS3 binding consensus sequence delineated by De Souza et al. (46). Therefore, we analyzed the consequence of substituting Tyr837 by phenylalanine on the inhibitory effect of SOCS3 on OSMR-mediated signaling. We did not detect a reduced sensitivity of signaling via a receptor complex containing the IL-5Rβ/OSMRY837F mutant toward SOCS3 expression compared with a complex containing the IL-5Rβ/OSMRwt (compare Fig. 3C, right, and Fig. 5A). This is in sharp contrast to the substi-

**FIGURE 6.** SOCS3 associates with JAK 1 in response to OSM. HepG2 cells were preincubated for 30 min with 10 μM MG132, followed by stimulation with the indicated amounts of OSM and IL-6, respectively, for the indicated time points. Lysates were prepared and subjected to immunoprecipitation with a polyclonal antibody against SOCS3. The immunoprecipitated complexes were separated by SDS-PAGE and analyzed by Western blot with an antibody specific for SOCS3 and tyrosine-phosphorylated STAT3, respectively, and reprobed with a monoclonal antibody against STAT3.

The specific OSMR complex is composed of gp130 and the OSMR, and it is well established that SOCS3 binds to Tyr(P)759 of gp130, thereby inhibiting signal transduction (15, 22). Therefore, we were interested to see whether the inhibitory effect of SOCS3 on OSM signaling is mediated via binding to gp130 alone or whether SOCS3 also interacts with the OSMR. In reporter gene assays, overexpressed SOCS3 down-regulated signaling mediated by an OSMR complex in which gp130 signal transduction and SOCS3 binding is prevented (IL-5Rα/gp130YFFFFF and IL-5Rβ/OSMR). Indeed, at low levels of SOCS3 expression, inhibition of the OSMR-mediated signaling comparable with that of gp130 was observed (Fig. 3C). In contrast to direct binding of SOCS3 to JAKs, the inhibitory mechanism of SOCS3 is known to involve receptor binding as mentioned before. Therefore, our results suggested an interaction of SOCS3 with the OSMR similar to the recruitment to gp130. Recently, the erythropoietin receptor (EpoR) sequence surrounding Tyr(P)429/Tyr(P)431 (LKpYlPylLVVS) has been described to interact with SOCS3 with high affinity (47). In the same study, the most important residues involved in specific binding to the SOCS3 SH2 domain, based on a model structure, were suggested to be the −2, +1, and +3-positions relative to the phosphorylated tyrosine residue. The OSMR contains a tyrosine motif (Tyr837/Tyr839) very similar to the described EpoR motif, PNYLYLLPT, in which the amino acids in positions −2, +1, and +3 also have hydrophobic side chains. It also fits quite well to the described SOCS3 binding consensus sequence delineated by De Souza et al. (46). Therefore, we analyzed the consequence of substituting Tyr837 by phenylalanine on the inhibitory effect of SOCS3 on OSMR-mediated signaling. We did not detect a reduced sensitivity of signaling via a receptor complex containing the IL-5Rβ/OSMRY837F mutant toward SOCS3 expression compared with a complex containing the IL-5Rβ/OSMRwt (compare Fig. 3C, right, and Fig. 5A). This is in sharp contrast to the substi-
tution of the SOCS3-recruiting tyrosine residue 759 in gp130, which results in a SOCS3-resistant gp130-mediated signal transduction (Fig. 5B, first two columns of results). Indeed, previous studies from our laboratory showed that this Y759F mutant of gp130 is only inhibited by SOCS3 when the concentrations of cotransfected SOCS3 cDNA are increased—100-fold (10 μg instead of 100 ng) (53). Looking for an alternative interaction motif for SOCS3 in the OSMR cytoplasmic region, we noticed to our surprise that the presence of the box 1/2 region of the OSMR is sufficient to mediate the inhibitory effect of SOCS3. All tyrosine motifs C-terminal to this region can be deleted without affecting the negative activity of SOCS3 (Fig. 5B). In an additional approach, we used biotinylated peptides encompassing phosphotyrosine motifs deduced from the cytoplasmic region of the OSMR as baits to immunoprecipitate SOCS3 expressed in HEK293T cells. None of the OSMR-based phosphotyrosine peptides bound SOCS3, whereas a strong recruitment was observed with the peptide encompassing the Tyr(P)759 motif of gp130 (Fig. 5C).

These data implied a potential direct binding of SOCS3 to the OSMR-associated JAKs at concentrations of SOCS3 that were not sufficient to allow direct binding to the gp130-associated JAKs. This is demonstrated by the fact that a receptor complex lacking the SOCS3 recruitment site in both gp130 intracellular chains (gp130Y759F) is not inhibited by SOCS3 at a concentration more than sufficient for inhibiting OSMR-mediated reporter gene induction (Fig. 5B, compare columns 1 and 2 with columns 3 and 4).

Indeed, we clearly found an interaction of SOCS3 and JAK1 in HepG2 cells stimulated with OSM; however, no convincing interaction of these two proteins was found in response to IL-6 (Fig. 6). To our knowledge, these are the first results showing an in vivo interaction between SOCS3 and JAK1 and imply that OSM- and IL-6-mediated signal transduction are negatively regulated by different mechanisms. Whereas negative regulation of IL-6-mediated signaling requires the recruitment of SOCS3 to gp130 via tyrosine 759, OSM-mediated signaling additionally results in a direct interaction of SOCS3 and JAK1.

There are two possible explanations for this observation. First, the difference in the association of JAK1 and SOCS3 in response to OSM and other stimuli might be explained by the strong induction of SOCS3 observed in response to OSM. In comparison with IL-6 the amount of SOCS3 induced by OSM is severalfold higher in human hematoma cells as well as murine fibroblasts (Fig. 1). Additionally, we routinely observed that the STAT3 phosphorylation levels induced by OSM returned to baseline within 2 h, whereas those induced by IL-6 were still measurable at 6 h post-OSM stimulation (Fig. 1B, columns 3 and 4).

Future studies are under way to corroborate this finding and prove that it holds true also for JAK1.

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