MECHANISMS OF SOCS3 PHOSPHORYLATION UPON IL-6 STIMULATION: CONTRIBUTIONS OF SRC- AND RECEPTOR TYROSINE KINASES

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Running Title: Mechanisms of SOCS3 phosphorylation

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The suppressors of cytokine signalling (SOCS) are negative feedback inhibitors of cytokine signal transduction. SOCS3 is a key negative regulator of IL-6 signal transduction. Furthermore, SOCS3 was shown to be phosphorylated upon treatment of cells with IL-2 and this has been reported to regulate its function and half-life. We set out to investigate whether SOCS3 phosphorylation may play a role in IL-6 signalling. Tyrosine phosphorylated SOCS3 was detected upon treatment of mouse embryonic fibroblasts (MEF) with IL-6. Interestingly, the observed SOCS3 phosphorylation does not require SOCS3 recruitment to phosphotyrosine pY759 of gp130 and the kinetics of SOCS3 phosphorylation do not match the activation kinetics of the Janus kinases. This suggests that other kinases may be involved in SOCS3 phosphorylation. Using Src and Janus kinase inhibitors as well as Src kinase deficient MEF cells, we provide evidence that Src kinases, which we found to be constitutively active in these cells, are involved in the phosphorylation of IL-6 induced SOCS3. In addition, we found that receptor tyrosine kinases (RTKs) such as PDGFR or EGFR can very potently phosphorylate IL-6 induced SOCS3. Taken together, these results suggest that SOCS3 phosphorylation is not a JAK-mediated phenomenon but is dependent on the activity of other kinases such as Src kinases or RTKs which can either be constitutively active or activated by an additional stimulus. Interleukin-6 (IL-6) is a cytokine which plays important roles in the coordination and regulation of immune responses. It was first described as B-cell stimulating factor-2 which induces differentiation and proliferation of B and T cells (1). Furthermore, IL-6 is the major mediator of acute phase proteins (APP) in rat hepatocytes (2-4). It is also implicated in several immune diseases such as rheumatoid arthritis and multiple myeloma (5).

IL-6 type cytokines transduce their signals via the JAK/STAT and MAPK pathways (6,7). Their common signal transducing receptor subunit, gp130, was shown to bind JAK1, JAK2 and TYK2 (8,9). Of these, JAK1 plays an essential role, because in cells lacking JAK1 IL-6 signal transduction is greatly impaired (10,11). Activation of the Janus kinases upon IL-6 stimulation induces receptor phosphorylation and subsequent recruitment of signalling proteins. Among these is STAT3 which binds to the four distal tyrosine motifs of gp130 (Y767, Y814, Y905 and Y915), becomes phosphorylated and translocates to the nucleus to induce IL-6 responsive genes (12-15). Tyrosine 759 of gp130 has been suggested to be involved in the inhibition of IL-6 signalling since the mutation of this tyrosine to phenylalanine enhances IL-6 signalling (16,17). It was demonstrated that Y759 can recruit both the tyrosine phosphatase SHP-2 and the feedback inhibitor suppressor of cytokine signalling 3 (SOCS3) (12,16,18,19). SHP-2 was shown to negatively regulate the IL-6 signalling pathway, but it was also reported that

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recruitment of SHP-2 to gp130 functions as an adaptor and leads to Ras/MAPK activation (20,21). SOCS3 belongs to the family of SOCS proteins, which have been shown to be induced by a number of cytokines and growth factors (22-24). The members of this protein family (CIS and SOCS1 to SOCS7) contain a central SH2 domain and a C-terminal SOCS box. SOCS3 is rapidly induced upon IL-6 stimulation and inhibits IL-6-mediated signalling in a classical feedback loop (18,25). The crucial role of SOCS3 for the inhibition of IL-6 signalling was recently confirmed in SOCS3 deficient macrophages (26-28). SOCS3 recruitment to the tyrosine motif Y759 of gp130 was shown to be required for its inhibitory action (18,19). Several mechanisms by which SOCS proteins regulate cytokine signalling have been proposed: SOCS1, and to some extent SOCS3, were found to inhibit the kinase activity of JAKs, by binding to the activation loop of the kinase (29,30). Another model for SOCS function proposes the targeting of signalling components to proteasome-dependent degradation (31-33). SOCS proteins were shown to form an E3-ubiquitin ligase complex with elonginC, elonginB, cullin2/5 and ring box protein-1 (34). It was reported that the C-terminal SOCS box of SOCS1 and SOCS3, which interacts with elonginC, is required for proteasomal degradation of SOCS binding partners such as TEL-JAK2, JAK2, Vav, insulin receptor substrate-1 and -2 (35-39).

SOCS3 is tyrosine phosphorylated upon IL-2, IL-3, erythropoietin (Epo), epidermal growth factor, platelet-derived growth factor and insulin stimulation (40-42). This modification occurs on Y204 and Y221 within the SOCS box and seems to be involved in regulation of the stability of SOCS3 (41,43). Furthermore it was reported that phosphorylated SOCS3 interacts with p120RasGAP to prolong ERK activation (41). The mechanism which underlies SOCS3 phosphorylation is not well understood. In previous studies it has been demonstrated that overexpression of Janus kinases or the Src kinase Lck leads to SOCS3 phosphorylation (40,41,43). However, the kinase which triggers SOCS3 phosphorylation upon cytokine stimulation has not been identified so far. We set out to investigate SOCS3 phosphorylation in the context of IL-6 signal transduction. We found IL-6 induced SOCS3 to be phosphorylated in MEF cells, but that Janus kinases are not responsible for the observed phosphorylation. We provide evidence that IL-6 induced SOCS3 can be phosphorylated by other kinases such as Src kinases or receptor tyrosine kinases (e.g. EGFR or PDGFR) in cells where these kinases are either constitutively active or have been activated by an additional stimulus.

EXPERIMENTAL PROCEDURES

Materials
The preparation of recombinant human IL-6 was performed by the method of Arcone et al. (44). The soluble IL-6Ra (sIL-6Ra) was prepared as previously described by Weiergräber et al. (45). Recombinant human Epo was a generous gift from Drs. B. Hilger and K.H. Sellinger (Roche, Mannheim, Germany). Murine OSM was obtained from R&D systems, human EGF and human PDGF-AB was purchased from Peprotech (London, UK). The gp130-derived peptides were described previously (18) and were kindly provided by Dr. J. Schneider-Mergener (Charité, Berlin, Germany). The proteasome inhibitor MG132 was obtained from Calbiochem (Darmstadt, Germany), the Src kinase inhibitor PP1 from Biomol (Hamburg, Germany) and the Janus Kinase Inhibitor 1 from Calbiochem (Darmstadt, Germany).

Plasmids
The plasmid pcDNA3-hSOCS3 was previously described (46). pcDNA3-Myc-SOCS3 F25A and pCMV2-Flag-SOCS3 were described previously (30). Murine cDNAs encoding the GST-tagged kinase domain of JAK2 (JAK2-JH1) and an inactive kinase domain where Y1007, Y1008 are mutated to phenylalanine (JAK2-JH1FF) were kindly provided by Dr. A. Yoshimura (Kyushu University, Fukuoka, Japan). The N-terminal GST-tag was replaced by a yellow fluorescent protein (YFP)-tag by transferring JAK2-JH1 into the previously described expression vector pEF-JAK2-YFP (47). pSVL-JAK1, pSVL-JAK2, pSVL-JAK3, pSVL-TYK2 were described previously (48-50). pME18S-SOCS3-wt, pME18S-SOCS3-Y204F, pME18S-SOCS3-221F and pME18S-SOCS3-FF were described previously (41).

The preparation of cDNAs encoding different variants of the chimeric receptors EpoR/gp130 was described previously (15). These constructs contain the extracellular part of the EpoR and the transmembrane and cytoplasmic parts of gp130. Chimeric receptor constructs EpoR/gp130 (YF4Y), EpoR/gp130 (YY4F) contain tyrosine to phenylalanine substitutions within the cytoplasmic part of the receptor as indicated. The receptors were subcloned into the pM5-retroviral.
vector based on the myeloproliferative sarcoma virus containing NRF IRES followed by cDNA encoding a GFP-Neo fusion protein.

Antibodies
For immunoprecipitations a SOCS3 rabbit antibody FA1017 (Fusion Antibodies Belfast, UK), a SOCS3 rabbit polyclonal antibody C005 (IBL, Hamburg, Germany) and a JAK1-HR785 rabbit polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) were used. For immunodetection, the following primary antibodies were used unless otherwise noted: SOCS3-M20 goat polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany), JAK1-HR785 rabbit polyclonal antibody, JAK2-C20 and JAK3-C21 (Santa Cruz Biotechnology, Heidelberg, Germany), TYK2-T20220 (BD, Heidelberg, Germany), Lck-FA1072 antibody (Fusion Antibodies Belfast, UK) phosphotyrosine-specific STAT3 (pY705STAT3) rabbit polyclonal antibody (Cell Signalling, Frankfurt, Germany), STAT3 mouse antibody (BD, Heidelberg, Germany), GFP mouse antibody (Santa Cruz Biotechnology, Heidelberg, Germany), phosphotyrosine-specific Src (pY-418) rabbit polyclonal antibody and a mixture of the phosphotyrosine mouse antibodies 4G10 (Upstate Biotechnology, Hamburg, Germany) and PY99 (Santa Cruz Biotechnology, Heidelberg, Germany). Horse radish peroxidase-conjugated secondary antibodies were obtained from DAKO (Hamburg, Germany).

Cell culture and transfection
COS-7 cells, Yes/Fyn deficient cells (Src++), Src/Yes/Fyn deficient MEF cells (SYF), SYF cells reconstituted with c-Src (SYF+c-Src), A431 (epidermoid cancer cell) and NIH-3T3 fibroblasts were purchased from ATCC. NIH-3T3 fibroblasts stably expressing wild type SOCS3 or mutant SOCS3 (SOCS3-FF) were described previously (41). The cell lines were cultivated in DMEM medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Seromed, Wien, Austria) and 1% Penicillin/Streptomycin (Gibco, Karlsruhe, Germany). Stable MEF cells were cultivated additionally in 1% neomycin (SIGMA, Taufkirchen, Germany). COS-7 cells were transfected by the diethylaminoethy-dextrane method as described previously (49). For stimulation, the cells were starved in medium without FCS and treated with 20 ng/ml IL-6 and 1 μg/ml sIL-6R, 20 ng/ml OSM, 7 U/ml Epo, 50 ng/ml EGF or 50 ng/ml PDGF-AB. The inhibitor MG132 was dissolved in DMSO and used at a concentration of 10 μM.

Retroviral infection of murine embryonic fibroblasts
Retroviral vectors pM5-EpoR/gp130 (6Y), pM5-EpoR/gp130 (YF4Y), pM5-EpoR/gp130 (YY4F) were introduced into murine embryonic fibroblasts. Briefly, retroviral vectors were transfected together with Ecopack packaging vector and gag-pol vector into Hek293T cells, after 48 h supernatants were collected and used in presence of polybrene (8 μg/ml) for infection of target cells. After 48 h transduced cells were subjected for G418 selection. The expression of the chimeric receptors was verified using Western blotting. A pool of positive cells was used for the experiments.

Immunoprecipitation
For immunoprecipitation of endogenous proteins about 3x10^7 cells were lysed in 500 to 800 μl lysis buffer (1% TritonX-100, 20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 10 mM NaF supplemented with 1 mM Na3VO4, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin and 10 μM MG132) for 30 min at 4°C. Insoluble material was removed by centrifugation, and cell lysates were precleared with protein A-Sepharose (Amersham, Freiburg, Germany) for 1 h at 4°C. After removal of the sepharose the lysates (1 to 3 mg protein) were incubated overnight with specific antibodies at 4°C. The immune complexes were bound to protein A-Sepharose for 1 h at 4°C. After centrifugation the beads were washed three times with washing buffer (0.1% TritonX-100, 20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4). The precipitated proteins were resolved by SDS-PAGE.

Peptide precipitation assay
COS-7 cells were transfected with pcDNA3-hSOCS3 (2 μg) and pEF-YFP-JAK2-JH1 or pEF-YFP-JAK2-JH1 (3 μg), respectively. Approximately 3 nM of the biotinylated peptides were immobilized by incubation with 5 mg of NeutrAvidin-coupled sepharose (Pierce, Bonn, Germany) in 100 μl lysis buffer PP (150 mM NaCl, 50 mM Tris/HCl, 0.1 mM EDTA, 10% glycerine, 0.5% Nonidet P-40, pH 8.0) per sample. For SOCS3 precipitation, transfected cells were lysed in lysis buffer PP supplemented with 1 mM Na3VO4, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin.
and MG132 and the total cell lysates were incubated with the immobilized peptides at 4°C overnight. Precipitates were washed three times with lysis buffer PP. The precipitated proteins were resolved by SDS-PAGE.

**Immunoblotting and immunodetection**
The electrophoretically separated proteins were transferred to a polyvinylidene difluoride PVDF membrane (Millipore, Eschborn, Germany) by semidry Western blotting method. Antigens were detected by incubation with specific primary antibodies and horse radish-peroxidase-coupled secondary antibodies (DAKO, Hamburg, Germany). The membranes were developed using the ECL detection system (Amersham Pharmacia Biotech, Freiburg, Germany). Quantification of Western blot signals was performed using the *Quantity one* software (BIORAD, München, Germany)

**RESULTS**

**SOCS3 phosphorylation is observed upon treatment of MEF cells with IL-6 or OSM**

In order to investigate whether endogenous SOCS3 can be phosphorylated upon IL-6 stimulation we treated 2fTGH, 2C4, HepG2, 293T and MEF cells with IL-6. In 2fTGH, 2C4, HepG2, and 293T cells, IL-6 potently induces the expression of SOCS3 (data not shown). However, we found detectable SOCS3 phosphorylation to occur only in MEF cells treated with IL-6 (Fig. 1A). Figure 1A (upper panels) shows the kinetics of SOCS3 phosphorylation in MEF cells treated with IL-6 and sIL-6R. Because we found that phosphorylation of SOCS3 destabilises the protein (43), the proteasome inhibitor MG132 was added to facilitate the detection of phosphorylated SOCS3 (pY-SOCS3). SOCS3 phosphorylation was seen after 60 min and persisted up to 180 minutes. The kinetics of phosphorylation exactly match those of SOCS3 protein expression. A control for which the cells were only treated with MG132 for 180 min (lane 7) shows that the inhibition of proteasomal degradation does not by itself lead to the accumulation of SOCS3 protein. To ascertain that the antibody FA1017 (Fusion Antibodies) precipitates the phosphorylated and non-phosphorylated forms of SOCS3 equally well, we compared this antibody with a SOCS3 antibody directed against the 18 N-terminal amino acids of SOCS3 (C005; IBL) (Fig. 1A, lower panels). As can be seen in figure 1A (lower panels; lanes 4 and 5) both antibodies FA1017 and C005 precipitate equal amounts of pY-SOCS3. The antibody C005 does not recognize the shorter SOCS3 isoform which lacks the 11 N-terminal amino acids (51).

We next checked whether SOCS3 phosphorylation could also be observed after stimulation of MEF cells with other IL-6-type cytokines. Because these cells express the OncostatinM (OSM) receptor on their cell surface, we compared IL-6 and OSM in their capacity to induce SOCS3 phosphorylation. As shown in Fig. 1B, pY-SOCS3 was also detected after treatment of MEF cells with OSM for 30 or 60 minutes.

**The kinetics of JAK activation and SOCS3 phosphorylation do not match**

It is well documented that SOCS3 is induced upon IL-6 stimulation via the JAK/STAT pathway and that the expression of this feedback inhibitor then leads to the inhibition of IL-6 mediated signalling (18,25-28).

To compare the activation kinetics of IL-6 signalling components such as JAK1 and STAT3 with SOCS3 phosphorylation, MEF cells were stimulated with IL-6/sIL-6R and SOCS3 was immunoprecipitated from the lysates (Fig. 2). SOCS3 expression was detectable at 30, 60 as well as 120 min and phosphorylation was visible at 60 and 120 min (Fig. 2, upper two panels). Endogenous JAK1 was also immunoprecipitated and its phosphorylation was monitored. Upon stimulation, JAK1 phosphorylation increased up to 30 min and then declined slowly until 120 min (Fig. 2, middle panels). The increase in STAT3 phosphorylation parallels the JAK1 phosphorylation but then pY-STAT3 decreases much more dramatically and is hardly visible at 60 min (Fig. 2, lower two panels). This decrease in STAT3 phosphorylation at 60 min correlated closely with the expression of the feedback inhibitor SOCS3. Thus, although JAK phosphorylation is still detectable at 60 and 120 minutes, the kinase is inhibited at this time. These results suggest that the Janus kinases may not be responsible for the observed SOCS3 phosphorylation.

**Recruitment to gp130 is not required for phosphorylation of SOCS3 upon IL-6 stimulation**

IL-6 induced SOCS3 needs to be recruited to the tyrosine motif pY759 within the signal transducing receptor chain gp130 in order to exert its inhibitory action (18,19). Furthermore, it was shown that the Janus kinases are
constitutively associated with this receptor chain (47,52). Thus the question arises whether SOCS3 recruitment to pY759 of gp130 is a prerequisite for SOCS3 phosphorylation. To address this question, MEFs were retrovirally transduced with chimeric receptors composed of the extracellular part of the EpoR and the intracellular part of gp130. This system allows mechanistical studies with receptor mutants independent of endogenous gp130. Three different stable cell lines were generated in which the chimeric receptors differ in their cytoplasmic regions (Fig. 3A). The cell line EpoR/gp130-6Y represents the wild type receptor in this system where all tyrosines in the intracellular part of gp130 are intact. In the cell line EpoR/gp130-YF4Y, the tyrosine Y759 of the SOCS3 recruiting motif VQYSTVVH is mutated to phenylalanine. The cell line EpoR/gp130-YY4F is a negative control where all STAT-recruitment sites are mutated to avoid SOCS3 expression. Comparable expression levels of the different chimeric receptors in the three cell lines were confirmed by immunoprecipitation and Western blotting (data not shown).

After Epo stimulation of the different cell lines, endogenous SOCS3 was immunoprecipitated and SOCS3 expression was analysed. Most interestingly, tyrosine phosphorylation was observed upon stimulation of both the wild type receptor as well as the mutant receptor (YF4Y) which can not recruit SOCS3 (Fig. 3A, upper panels). As a control, STAT3 activation was monitored. The stronger induction of SOCS3 upon stimulation of the chimeric receptor EpoR/gp130-YF4Y correlated with the expected stronger STAT3 activation observed in this cell line. This is due to the fact that neither SOCS3 nor the tyrosine phosphatase SHP-2, which has also been shown to inhibit IL-6 signalling via the Y759 in gp130 (12,20), can bind to phenylalanine. The cell line EpoR/gp130-YY4F is a negative control where all STAT-recruitment sites are mutated to avoid SOCS3 expression. Comparable expression levels of the different chimeric receptors in the three cell lines were confirmed by immunoprecipitation and Western blotting (data not shown).

SOCS3 phosphorylation does not disrupt the function of the SH2 domain

Having found that SOCS3 phosphorylation appears to occur independently of receptor recruitment, we were interested to determine whether the phosphorylation of SOCS3 would on the other hand affects its ability to bind to gp130, which is dependent on the SOCS3 SH2 domain (20). To test this, a peptide precipitation assay was performed in which biotinylated peptides encompassing the tyrosine motif Y759 of gp130 in a phosphorylated or non-phosphorylated form were used to precipitate SOCS3 and pY-SOCS3. To obtain the modified or non-modified form of SOCS3, it was coexpressed together with a YFP-tagged, constitutively active (JAK2-JH1) or inactive (JAK2-JH1FF) kinase domain of JAK2. This hyperactive kinase domain is able to potently phosphorylate SOCS3 on Y204 and Y221 if it is overexpressed (43). Figure 4A shows that SOCS3 is efficiently phosphorylated upon overexpression of JAK2-JH1 whereas the inactive mutant JAK2-JH1FF is not able to induce SOCS3 phosphorylation (lanes 1 and 2). Lanes 3 and 4 show controls lacking SOCS3 or JAK2-JH1, respectively. The lysates were then incubated with the phosphorylated or non-phosphorylated peptides. Following peptide precipitation with NeutrAvidine-sepharose, coprecipitation of phosphorylated and non-phosphorylated SOCS3 was monitored using a phosphotyrosine- and a SOCS3-specific antibody. Figure 4B demonstrates that both phosphorylated and non-phosphorylated SOCS3 bound to the phosphorylated peptide pY759 (lanes 1 and 2). As expected, the control lanes with a non-phosphorylated Y759 peptide (lanes 5 to 8) did not show significant SOCS3 binding.
Thus, tyrosine phosphorylation of SOCS3 does not disrupt the function of the SH2 domain.

SOCS3 phosphorylation observed upon IL-6 stimulation can be blocked by the Src kinase inhibitor PP1

Since SOCS3 phosphorylation was first shown to occur upon treatment of cells with IL-2 (40), a cytokine known to activate the Src kinase Lck, we investigated whether Src kinases could be responsible for the observed SOCS3 phosphorylation in MEF cells. For this, we stimulated MEF cells stably expressing EpoR/gp130-YF4Y with Epo in the presence of increasing amounts of the Src kinase inhibitor PP1 and the Janus kinase inhibitor JAK inhibitor 1. SOCS3 was precipitated from the lysates and subjected to Western blot analysis (Fig. 5, upper two panels). As a control, STAT3 phosphorylation was also monitored (Fig. 5, lower two panels). We found SOCS3 phosphorylation to be dramatically decreased upon treatment of the cells with the Src kinase inhibitor PP1 at concentrations of 5, 10 and 20 μM (lanes 3-6). STAT3 activation was not affected, showing that the inhibitor did not block the JAK/STAT pathway. Treatment with PP1 at a concentration of 1 μM already potently inhibited SOCS3 phosphorylation (data not shown). In sharp contrast, the JAK inhibitor 1 did not affect SOCS3 phosphorylation at concentrations of 50, 100 and 150 nM, whereas STAT3 phosphorylation was dose-dependently reduced (lanes 3, 7, 8 and 9). Higher concentrations of this inhibitor were usually avoided to prevent non-specific effects. Taken together, these findings indicate that SOCS3 phosphorylation may primarily be mediated by kinases of the Src family.

The Src kinase Lck potently phosphorylates SOCS3

The isolated kinase domain of JAK2 (JAK2-JH1) is able to potently phosphorylate SOCS3 if both proteins are cotransfected in COS-7 cells ([43] and Figure 4). It is known that the activity of the isolated kinase domain of JAK2 is about 50 fold increased if compared to the kinase domain within the full length protein (53). We next checked whether the JAK2-JH1 mediated SOCS3 phosphorylation could be dependent on Src kinases. COS-7 cells were cotransfected with expression vectors which encode SOCS3 and JAK2-JH1. After 48h, the cells were incubated with increasing amounts of PP1 inhibitor. Figure 6A shows that the JAK2-JH1 induced SOCS3 phosphorylation is not affected by the Src kinase inhibitor PP1, suggesting that the highly active JAK2-JH1 is able to directly phosphorylate SOCS3 upon overexpression.

In previous studies, it was also shown that overexpression of full length JAK1 can induce SOCS3 phosphorylation (40,41,43). To compare the potency of full length JAK1 and Lck to phosphorylate SOCS3, we cotransfected SOCS3 with either Lck or JAK1 expression vectors (Fig. 6B). After 48h, the constitutive activity of the kinases was blocked by treatment of the cells with increasing amounts of JAK inhibitor 1 or PP1. Overexpression of JAK1 leads to the constitutive activation of STAT3 (Fig. 6B, lane 2). This activation can be blocked by the JAK inhibitor 1 (lanes 5 and 6) but not by the Src kinase inhibitor PP1 (lane 3 and 4). Although JAK1, at these expression levels, was able to potently phosphorylate STAT3, SOCS3 tyrosine phosphorylation could not be observed (upper panel, lanes 2-6). In contrast, very strong SOCS3 phosphorylation was detected upon overexpression of Lck (lane 7) which was inhibited by PP1 (lanes 8 and 9) but not by the JAK Inhibitor 1 (lane 10 and 11). The fact that the JAK inhibitor 1 does not influence SOCS3 phosphorylation indicates that Lck may phosphorylate SOCS3 directly. The absence of pY-SOCS3 in cells expressing JAK1 at levels sufficient to induce a constitutive STAT3 phosphorylation supports the idea that Janus kinases do not contribute to SOCS3 phosphorylation under physiological conditions although they may do so if sufficiently overexpressed. To check this hypothesis, we coexpressed SOCS3 with increasing amounts of JAK1 (Fig. 6C). Figure 6C shows that although JAK1 autophosphorylation is easily detectable at the lowest JAK1 expression level (lane 2) SOCS3 phosphorylation is only visible at the highest JAK1 expression level (lane 6). This finding confirms our previous observation that JAK1 overexpression can lead to SOCS3 phosphorylation (40,41,43) but further suggests that JAK1 is not the kinase which phosphorylates SOCS3 under physiological conditions.

We next investigated the potency of the different Janus kinase family members to phosphorylate SOCS3 upon overexpression of the kinases. To avoid partial inhibition of the Janus kinases by SOCS3, we cotransfected a SOCS3 construct carrying a mutation in the kinase inhibitory region (SOCS3-F25A) together with the different kinases. It was previously shown that this mutation disrupts the inhibitory function of
SOCS3 (30). Although the expression levels of the different kinases cannot be compared directly, Figure 6D shows that JAK levels which are sufficient to induce autophosphorylation do not necessarily lead to SOCS3 phosphorylation. In cells transfected with Lck, SOCS3-F25A was well phosphorylated. Interestingly, cotransfection of JAK3 reduced SOCS3 expression levels. The underlying mechanism is currently unclear.

As the tyrosine residues Y204 and Y221 in SOCS3 were shown to be phosphorylated upon IL-2 stimulation as well as upon overexpression of JAK1 or the isolated JAK2-JH1 domain (41,43) we asked whether Lck would also lead to the phosphorylation of these two tyrosine residues. COS-7 cells were cotransfected with Lck and different SOCS3 constructs encoding for wt-SOCS3, SOCS3-Y204F, SOCS3-Y221F or the double mutant SOCS3-FF. Figure 6E shows that Lck phosphorylates both tyrosine Y204 and Y221 (lanes 5 and 4). Tyrosine Y204 is more prominently phosphorylated than tyrosine Y221. The double mutant SOCS3-FF, where both tyrosines Y204 and Y221 are mutated to phenylalanine, is not phosphorylated (lane 6).

SOCS3 phosphorylation upon IL-6 stimulation is impaired in Src kinase deficient MEF cells
To further explore the hypothesis that Src kinases may be involved in the phosphorylation of SOCS3, we took advantage of Src-deficient MEF cells (called SYF cells) that lack all three Src kinase family members (Src, Yes and Fyn) expressed in wild type MEF cells. As a control, we used MEF cells in which only Yes and Fyn were depleted but that still express Src (Src++ cells) and SYF cells which have been reconstituted with c-Src (SYF+c-Src) (Fig. 7A and B, TCL, middle panel). STAT3 activation is comparable in all three cell types (Fig. 7A and B, TCL, lower two panels). SOCS3 phosphorylation upon IL-6 stimulation of MEF cells thus seems to occur constitutively in a Src kinase dependent manner. However, our findings show that Src kinases are not solely responsible for the constitutive phosphorylation of IL-6 induced SOCS3 and that other kinases also contribute to this phosphorylation.

EGF as well as PDGF induce strong phosphorylation of IL-6 induced SOCS3
As we found that Src kinases are not exclusively responsible for the constitutive phosphorylation of IL-6 induced SOCS3 (Fig. 7), we investigated whether other kinases could also efficiently phosphorylate SOCS3 in a cross-talk event. It has been reported that stimulation of cells with EGF or PDGF induces SOCS3 expression and phosphorylation (41). But the induction of SOCS3 upon RTK activation (e.g. PDGFR) is generally much lower than cytokine mediated SOCS3 expression. This correlates with their weaker ability to activate STAT factors (Fig. 8A). Since IL-6 induces SOCS3 very potently and as our results show that SOCS3 phosphorylation is not an IL-6 mediated event we were interested to investigate whether RTKs are also able to efficiently phosphorylate IL-6 induced SOCS3. We prestimulated A431 cells for the indicated times (0, 20, 60 min) with IL-6 and subsequently treated the cells with EGF for 15 min before they were harvested. Figure 8B shows that IL-6 induced SOCS3 (60 min...
stimulation with IL-6, lane 6) is strongly phosphorylated by the short EGF pulse. The same was observed when NIH-3T3 fibroblasts were prestimulated with IL-6 and then treated with PDGF for 15 min. While IL-6 stimulation alone only leads to a weak phosphorylation of SOCS3 (lanes 5 in Fig. 8B), which is probably due to some basal kinase activity in these cells (as we observed in MEF cells), an additional short stimulation with EGF or PDGF results in a strong phosphorylation of the IL-6 induced SOCS3.

As RTKs such as PDGFR were shown to activate Src kinases (55) we next investigated whether the PDGF-mediated phosphorylation of IL-6 induced SOCS3 is Src kinase dependent. For this, MEF and SYF cells were first stimulated with IL-6 for 90 min to induce SOCS3. Then, the cells were treated with PDGF for 15 min to phosphorylate SOCS3. As we observed before (Figure 7), the weak constitutive phosphorylation of SOCS3 detected in MEF cells upon IL-6 stimulation (Figure 8C, lane 2) is strongly reduced in the SYF cells (lane 5), arguing that Src kinases are involved in this process. However, Figure 8C clearly shows that PDGF potently phosphorylates the IL-6 induced SOCS3 both in MEF cells and in the Src kinase-deficient SYF cells (Figure 8C, lanes 3 and 6), demonstrating that Src kinases are not required for PDGF mediated SOCS3 phosphorylation. This shows that in addition to Src kinases, other tyrosine kinases can phosphorylate IL-6 induced SOCS3.

To further assess the extent of SOCS3 phosphorylation upon stimulation of cells with IL-6 or PDGF, we treated NIH-3T3 cells stably expressing wild type SOCS3 (SOCS3-wt) or mutant SOCS3 (SOCS3-FF) with IL-6 or PDGF. As shown in Figure 8D, SOCS3 can be constitutively phosphorylated in SOCS3-wt expressing cells (lane 1) and does not increase upon stimulation with IL-6 (lanes 2-4). Upon treatment with PDGF (lanes 5-7), a clear increase in SOCS3 phosphorylation is observed. In contrast, both the constitutive as well as the PDGF induced phosphorylation of SOCS3 is lost in cells expressing the double tyrosine mutant SOCS3-FF. Together with the data presented before this suggests that SOCS3 phosphorylation is not relevant in JAK mediated IL-6 signalling but may become relevant during cross-talk events involving constitutively active kinases or simultaneous treatment of cells with growth factors such as PDGF or EGF.

**DISCUSSION**

SOCS3 was shown to be a key negative regulator of IL-6 signal transduction (18,25-28). It is tyrosine phosphorylated on two tyrosine residues within the C-terminal SOCS box upon stimulation with cytokines and growth factors such as IL-2, IL-3, Epo, EGF and PDGF (40,41). The mechanism which underlies this phosphorylation is not well understood. In previous studies, it has been demonstrated that overexpression of Janus kinases or the Src kinase Lck can lead to SOCS3 phosphorylation. However, the kinase which triggers SOCS3 phosphorylation under physiological conditions has not been identified so far.

We set out to investigate whether SOCS3 phosphorylation may play a role in IL-6 signalling. Tyrosine phosphorylated SOCS3 was detected upon treatment of MEFs with IL-6 and OSM (Fig. 1). Further studies showed that the observed phosphorylation did not require SOCS3 recruitment to the receptor and that the kinetics of SOCS3 phosphorylation did not match the activation kinetics of the Janus kinases (Figs. 2 and 3). These results indicated that Janus kinases may not play a role in physiological SOCS3 phosphorylation.

As IL-2, the first cytokine for which SOCS3 phosphorylation was reported, activates both Janus kinases and the Src kinase family member Lck (56,57), we checked whether Src kinases could be mediating SOCS3 phosphorylation. Using inhibitors, we found that a Src kinase but not a Janus kinase inhibitor was able to suppress the phosphorylation of IL-6 induced SOCS3 (Fig. 5).

As Janus kinases were found to phosphorylate SOCS3 upon overexpression (40,41,43), we cotransfected SOCS3 with different Janus kinases or Lck. Lck potently phosphorylated SOCS3, whereas cotransfection of JAKs did not show prominent SOCS3 phosphorylation. Figure 6 indicates that only highly overexpressed Janus kinases lead to a detectable SOCS3 phosphorylation. To back up the studies involving kinase inhibitors, we took advantage of Src kinase deficient MEF cells (Fig. 7). In the cell line (Src−/−) lacking two of the three Src kinases expressed in MEF cells, namely Yes and Fyn, and in a Src deficient cell line in which c-Src has been reconstituted (SYF+c-Src) SOCS3 phosphorylation could be detected following the induction of SOCS3 by IL-6. In contrast, SOCS3 phosphorylation was reduced in SYF cells, where Src, Yes and Fyn are depleted. This
confirms that Src kinases are involved in the phosphorylation of SOCS3 in MEF cells stimulated with IL-6. We detected a constitutive Src kinase activation in these cells which was not increased upon treatment with IL-6 (Fig. 7). This is in line with a previous report which shows that Src is constitutively active in Src++ cells (54). Our results suggest that the observed phosphorylation of SOCS3 in MEF cells is not an IL-6 mediated phenomenon but is rather due to cross-talk events involving constitutively active Src kinases. The residual SOCS3 phosphorylation remaining in SYF cells indicates that other kinases may also be able to contribute to SOCS3 phosphorylation. For example, a short stimulation with EGF and PDGF leads to a strong phosphorylation of IL-6 induced SOCS3 in A431 and NIH-3T3 cells, respectively (Fig. 8B). Using the Src kinase deficient cells we could further show that the PDGF mediated phosphorylation of IL-6 induced SOCS3 is Src kinase independent (Fig. 8C).

Taken together, the observations indicate that even though the IL-6 driven JAK/STAT pathway strongly induces SOCS3 it does not mediate its phosphorylation. SOCS3 is rather phosphorylated by constitutively active kinases, such as Src kinases in MEF cells, or by another extracellular stimulus, such as EGF and PDGF. In this case, SOCS3 phosphorylation may thus become relevant during cross-talk events.

Src kinases and RTKs have been implicated in the development of numerous human cancers and are constitutively active in many cells. c-Src for example has been found to be highly activated in colon cancers, especially in metastatic liver cancers (58,59). Furthermore, in multiple myeloma cells, IL-6 was reported to activate the Src kinase Hck, which was proposed to mediate the proliferative effects of gp130 (60). As IL-6 is the most important growth factor involved in the proliferation of multiple myeloma cells, it would be interesting to determine whether SOCS3 phosphorylation contributes to malignant growth. Our finding that constitutive activation of Src kinases and the activation of RTKs lead to the phosphorylation of SOCS3 protein induced by cytokines (e.g. IL-6) indicates that phosphorylation of SOCS3 may contribute to the oncogenic potential of these kinases. This could be achieved via several mechanisms. As phosphorylated SOCS3 was reported to inhibit the Ras inhibitor p120RasGAP (41), one possible mechanism could be a prolonged Erk activation leading to increased proliferation of the cancer cells. Furthermore, SOCS3 stability as well as its interaction with the E3-ubiquitin ligase component elonginC was shown to be reduced upon phosphorylation of SOCS3 (43). This may impair the postulated E3-ubiquitin ligase function of SOCS3 and thus the degradation of proteins interacting with SOCS3. For example, a similar mechanism is involved in the development of the VHL syndrome. The VHL tumor suppressor protein, which is also part of an E3 ubiquitin ligase complex containing elonginB, elonginC, cullin2 and ring box protein-1 interacts with elonginC via its SOCS box (61-63). Formation of this complex stabilises the VHL protein and mutations in the SOCS box disrupting the interaction with elonginC, were shown to abolish its E3-ubiquitin ligase function leading to impaired degradation of recruited HIF-1α (64,65). Such mutations were found in patients with dominant inherited VHL syndrome, characterized by the predisposition to develop various benign and malignant tumors (66,67). In a very recent publication, a similar mechanism was also postulated for the E3-ubiquitin ligase function of SOCS1 (68). The authors reported that v-Abl induced modifications of SOCS1 disrupt its binding to elonginC and may thus impair SOCS1-dependent degradation of Janus kinases. In addition, pY-SOCS3 may also contribute to disease via another mechanism. As phosphorylation of SOCS3 decreases its half-life, the levels of SOCS3 induced upon stimulation with cytokines such as IL-6 could be reduced in cancer cells where Src kinases or RTKs are highly activated. This could lead to enhanced and prolonged cytokine signals and contribute to the malignant transformation of cells.

This paper strongly indicates that cytokine driven JAK activation is not involved in the physiological phosphorylation of SOCS3. Phosphorylation of SOCS3 seems to be more relevant during cross-talk events in which cytokines induce high levels of SOCS3 protein that in turn is then strongly phosphorylated in response to other extracellular stimuli or via constitutively active kinases. Our data clearly suggest that such a cross-talk leads to much higher cellular levels of phosphorylated SOCS3 than a single cytokine or growth factor stimulus. The effects of this high SOCS3 phosphorylation on the different signalling pathways involved in such a cross-talk event (e.g. IL-6- and RTK-induced signalling pathways) remain unclear and are currently under investigation.
FOOTNOTES
This work has been supported by the Deutsche Forschungsgemeinschaft (DFG; Bonn, Germany) and the Fonds der Chemischen Industrie (Frankfurt am Main, Germany).

The abbreviations used are: EGF, epidermal growth factor; Epo, erythropoietin; ERK, extracellular signal regulated kinase; GAP, GTPase activating protein; gp, glycoprotein; GST, glutathione S-transferase; IL, interleukin; JAK, Janus kinase; JH1, JAK homology region 1; KIR, kinase inhibitory region; Lck, lymphocyte kinase; MAPK, mitogen activated protein kinase; MEF, mouse embryonic fibroblasts; PDGF, platelet derived growth factor; pY, phosphotyrosine; R, receptor; RTK, receptor tyrosine kinase; SHP, SH2 domain containing protein tyrosine phosphatase 2; SOCS, suppressors of cytokine signaling; Src, Rous sarcoma; STAT, signal transducers and activators of transcription; VHL, Von Hippel-Lindau; YFP, yellow fluorescent protein

REFERENCES
FIGURE LEGENDS

Figure 1: SOCS3 tyrosine phosphorylation occurs upon treatment of MEF cells with IL-6 or OSM
A) Upper panels: MEF cells were stimulated with IL-6 (20 ng/ml) and sIL-6R (1 μg/ml) for the indicated times in the presence of MG132 [10μM]. SOCS3 was immunoprecipitated and probed with a pY and a SOCS3 antibody. Lower panels: MEF cells were stimulated with IL-6 and sIL-6R in the presence of the proteasome inhibitor MG132. SOCS3 was immunoprecipitated with the SOCS3 antibodies FA1017 (Fusion Antibodies; Belfast, UK) or C005 (IBL, Hamburg, Germany). Precipitates were probed with a pY antibody and SOCS3 counterstaining was performed with a SOCS3 antibody. B) MEF cells were stimulated with IL-6 (20 ng/ml) and sIL-6R (1 μg/ml) or OSM (20 ng/ml) for the indicated times in the presence of MG132. After immunoprecipitation of SOCS3, the precipitates were detected with a pY and a SOCS3 antibody.

Figure 2: Activation kinetics of SOCS3, JAK1 and STAT3 upon stimulation of MEF cells with IL-6
MEF cells were stimulated with IL-6 and sIL-6R for the times indicated in the presence of MG132. SOCS3 and JAK1 were immunoprecipitated from total cell lysates. Detection of SOCS3 was performed using a pY and a SOCS3 antibody. JAK1 phosphorylation was detected by using pY antibody mixture pY99/4G10 and the counterstaining was performed with a JAK1 specific antibody. STAT3 protein and tyrosine phosphorylation was detected from total cell lysates using specific pY705STAT3 and STAT3 antibodies.

Figure 3: Recruitment of SOCS3 to gp130 is not required for SOCS3 tyrosine phosphorylation.
A) MEF cells stably expressing different EpoR/gp130 receptor chimeras were stimulated with Epo (7 U/ml) in the presence of MG132 for the indicated times. SOCS3 precipitates were immunoblotted with a pY and a SOCS3 antibody. STAT3 activation and STAT3 protein levels were detected out of total cell lysates using a pY705STAT3 and a STAT3 antibody. B) EpoR/gp130-YF4Y expressing MEF cells were stimulated with Epo for the indicated times in the presence of MG132 (lanes 5-8) or DMSO (lanes 1-4). SOCS3 analysis was performed as described above. The amount of phosphorylated SOCS3 versus total SOCS3 protein was compared by quantification of the 60 min time point (lanes 3 and 7) using the Quantity one software (bar diagram).

Figure 4: SOCS3 phosphorylation does not abrogate the function of the SOCS3 SH2 domain
A) COS-7 cells were transiently cotransfected with expression vectors coding for SOCS3 (pcDNA3-hSOCS3, 2 μg) and YFP-tagged active JAK2-JH1wt or inactive JAK2-JH1FF (3 μg), respectively. The cells were incubated with MG132 for 4 h prior to lysis. Phosphorylation of SOCS3 and JAK2-JH1wt was detected from total cell lysates (TCL) using a mixture of phosphotyrosine specific antibodies (pY99/4G10). Counterstainings for SOCS3 and the YFP-JAK2 constructs was performed using a SOCS3 or a YFP-specific antibody. B) Peptide precipitation assay using a phosphorylated or non-phosphorylated peptide. The biotinylated peptides pY759 and Y759 were incubated with COS-7 lysates and were then precipitated with NeutrAvidine-Sepharose. The precipitates were resolved by SDS-PAGE and SOCS3 coprecipitation was investigated using a pY and a SOCS3 specific antibody.

Figure 5: SOCS3 phosphorylation upon IL-6 stimulation is inhibited by the Src kinase inhibitor PP1
MEF cells which stably express the chimeric receptor EpoR/gp130-YF4Y were stimulated with Epo for the indicated times in the presence of MG132. Cells stimulated for 120 min were then incubated with increasing amounts of PP1 or JAK Inhibitor 1. SOCS3 was immunoprecipitated from lysates and SOCS3 phosphorylation was detected with a pY specific antibody. SOCS3 counter staining was performed using a SOCS3 antibody. STAT3 activation was detected from lysates with specific pY705STAT3 and STAT3 antibodies.
Figure 6: The Src kinase Lck potently phosphorylates SOCS3
A) COS-7 cells were cotransfected with expression vectors encoding for SOCS3 (pCMV2-Flag-SOCS3, 2 μg) and YFP-JAK2-JH1 (3 μg) or with empty vector, respectively. Cells were incubated with MG132 for 4 h and and increasing amounts of PP1 were added for 1 h. The detections were carried out using total cell lysates. SOCS3 phosphorylation and SOCS3 protein were detected from the lysates using a specific pY and a SOCS3 antibody. JAK2-JH1 activation was detected using an antibody which specifically detects the phosphorylated tyrosines Y1007 and Y1008 in the activation loop of the kinase (JAK2-JH1pYpY). JAK2-JH1 counterstaining was performed using a YFP-antibody.
B) COS-7 cells were transfected with expression vectors encoding for SOCS3 (pCMV2-Flag-SOCS3, 0.5 μg) and JAK1 or Lck (3 μg), respectively. Cells were incubated with MG132 for 4 h and treated with increasing amounts of PP1 and JAK Inhibitor 1 for 1 h. pY-SOCS3, SOCS3, phosphorylated STAT3, JAK1 and Lck were detected from total cell lysates using specific antibodies recognizing pY, SOCS3, pY705STAT3, JAK1 and Lck, respectively.
C) COS-7 cells were cotransfected with an expression vector encoding for SOCS3 (pCMV2-Flag-SOCS3, 1 μg) and increasing amounts of an expression vector encoding for Jak1 as indicated. Cells were incubated with MG132 for 4 h prior to lysis. Phosphorylated Jak1 and SOCS3 were detected using a pY-antibody. SOCS3 and Jak1 were detected with specific antibodies recognizing SOCS3 and Jak1.
D) COS-7 cells were transfected with expression vectors coding for JAK1, JAK2, JAK3, TYK2 or Lck (3 μg) together with the SOCS3-F25A mutant (0.5 μg). Phosphorylation of SOCS3 was checked with a pY and a SOCS3 antibody. Phosphorylation of the kinases was assessed with a pY antibody and their expression was monitored using antibodies against JAK1, JAK2, JAK3, TYK2 and Lck.
E) COS-7 cells were transfected with expression vectors coding for SOCS3-wt, SOCS3-Y204F, SOCS3-Y221F and SOCS3-FF (1 μg) together with Lck (1 μg). Phosphorylation and expression of SOCS3 and Lck was checked with a pY, a SOCS3 (C005) and an Lck antibody.

Figure 7: SOCS3 phosphorylation is strongly reduced in Src kinase deficient cells
SYF (Src, Yes and Fyn deficient MEFs) and Src++ cells (Yes and Fyn deficient MEFs) (A) or SYF and SYF+c-Src cells (SYF cells reconstituted with c-Src) (B) were stimulated with IL-6 and sIL-6R in the presence of MG132. SOCS3 immunoprecipitation was performed with a SOCS3 antibody. Precipitates were blotted and detected with a pY and a SOCS3 antibody. Activated Src kinase and phosphorylated STAT3 were detected from total cell lysates from the same experiment using the phosphospecific antibodies pY418Src and pY705STAT3. To check for equal protein amounts in the samples a STAT3 counter staining was performed using a STAT3 specific antibody. The amount of phosphorylated SOCS3 in the different cell lines was compared by quantification of the 90 min time point using the Quantity one software (bar diagram). The values for pY-SOCS3 were normalised to SOCS3 expression and the value obtained in SYF cells was set to 1.

Figure 8: IL-6 induced SOCS3 is strongly phosphorylated upon stimulation with RTKs
A) NIH-3T3 fibroblasts were stimulated with IL-6/sIL-6R or PDGF for the indicated times. SOCS3 immunoprecipitation was performed with a SOCS3 antibody. Precipitates were blotted and detected with SOCS3 antibody. STAT3 activation was detected from total cell lysates using the phosphospecific antibody pY705STAT3 and a STAT3 antibody. B) Human epidermoid cancer cells (A431) and NIH-3T3 fibroblasts were prestimulated for the indicated times with IL-6 alone or with IL-6/sIL-6R in the presence of MG132. Subsequently, a 15 min stimulation with 50 ng/ml EGF (A431) or 50 ng/ml PDGF-AB (NIH-3T3) was performed. IL-6 induced SOCS3 was immunoprecipitated with a SOCS3 antibody. Precipitates were blotted and detected with a pY and a SOCS3 antibody. C) MEF and SYF cells were treated with MG132 and then prestimulated with IL-6 and sIL-6R for 90 min or were left untreated. Subsequently, a second stimulation with PDGF-AB (15 min) was performed. SOCS3 was immunoprecipitated using a SOCS3 antibody. Precipitates were blotted and detection was done with a pY and a SOCS3 antibody. D) 3T3 cells stably expressing SOCS3-wt or SOCS3-FF were stimulated with IL-6 and sIL-6 or PDGF-AB for the indicated times. SOCS3 was immunoprecipitated with a SOCS3 antibody and the detection was performed using a pY and a SOCS3 antibody.
Figure 1:

A

MG132

IL-6/sIL-6R

IP: SOCS3

WB: pY

pY-SOCS3

15 15 30 60 120 180 180 min

0 15 30 60 120 180 0 min

WB: SOCS3

B

IL-6/sIL-6R

OSM

IP: SOCS3

WB: pY

pY-SOCS3

0 15 30 60

15 30 60 min

WB: SOCS3
Figure 2

IL-6/sIL-6R 0 5 15 30 60 120 min

IP: SOCS3

| WB: pY | pY-SOCS3 |
| WB: SOCS3 |

IP: JAK1

| WB: pY | pY-JAK1 |
| WB: JAK1 |

TCL

| WB: pY-STAT3 | WB: STAT3 |
Figure 3

A

6Y

Y683

Y759

Y767

Y814

Y905

Y915

EpoR

gp130

YF4Y

Y683

F759

F767

Y814

F905

F915

YY4F

Y814

STAT3

Y905

F915

Epo

0 30 60 120 180 240 min

WB: pY

WB: pY-SOCS3

WB: SOCS3

WB: STAT3

IP: SOCS3

TCL

B

- MG132

+ MG132

Epo

0 30 60 180 min

WB: pY

WB: pY-SOCS3

WB: SOCS3

60 min Epo stimulation

relative band intensity
**Figure 4**

### A

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<td>-</td>
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**WB:** pY

\[\text{pY-JAK2} \quad \text{JH1}\]

\[\text{pY-SOCS3}\]

**WB:** GFP/YFP

\[\text{JAK2} \quad \text{JH1}\]

**WB:** SOCS3

1 2 3 4

### B

#### peptide precipitation

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**WB:** pY

\[\text{pY-SOCS3}\]

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**WB:** SOCS3

1 2 3 4 5 6 7 8
Figure 5

Epo  0  30  120  min

PP1 [μM]  JAK Inhibitor 1[nM]
- - -  5  10  20  50  100  150

IP: SOCS3

WB: pY
← pY-SOCS3

WB: SOCS3

TCL

WB: pY-STAT3

WB: STAT3
Figure 6

A

SOCS3

JAK2-JH1

PP1 [μM]

2.5 5 10

WB: pY  pY-SOCS3

WB: SOCS3

WB: pYpY-JAK2 JH1

TCL

WB: GFP/YFP  JAK2 JH1

B

SOCS3

JAK1

Lck

PP1 [μM]  JAK-Inh1 [nM]

2.5 5 100 500

WB: pY  pY-SOCS3

WB: SOCS3

WB: pY-STAT3

WB: JAK1

WB: Lck
Figure 6

C

SOCS3

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WB: pY

pY-Jak1

WB: pY-SOCS3

WB: SOCS3

1 2 3 4 5 6

WB: pY

pY-Jak1

WB: Jak1

TCL

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Figure 6

D

SOCS3-F^{25A}

- JAK1 JAK2 JAK3 TYK2 Lck

WB: pY

WB: pY-JAKs

WB: pY-Lck

WB: JAK1

WB: JAK2

WB: JAK3

WB: TYK2

WB: Lck

WB: pY

pY-SOCS3

WB: SOCS3

E

Lck

- WT WT Y204F Y224F FF

SOCS3

WB: pY

pY-Lck

WB: Lck

WB: pY

pY-SOCS3

WB: SOCS3

1 2 3 4 5 6
Figure 7

A

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<td></td>
</tr>
<tr>
<td>90</td>
<td></td>
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**IP: SOCS3**

**WB: pY-SOCS3**

**WB: SOCS3**

**90 min IL-6 stimulation**

rel. pY-SOCS3 normalised to SOCS3 expression

<table>
<thead>
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**Src++**

**SYF**

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**WB: pY-Src**

**WB: pY-STAT3**

**WB: STAT3**
Figure 7

**B**

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<td>0 30 90 min</td>
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<td><em>p</em>-soCS3</td>
<td><em>p</em>-soCS3</td>
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<tr>
<td></td>
<td>WB: <em>p</em>-Y</td>
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90 min IL-6 stimulation

rel. *p*-Y-soCS3 normalised to SOCS3 expression

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<td>0 30 90 min</td>
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Figure 8

A

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- TCL
  - WB: pY-STAT3
  - WB: STAT3
  - IP: SOCS3 WB: SOCS3

B

**A431 cells**

1st stimulation: IL-6
2nd stimulation: EGF

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<td>2nd stimulation: EGF</td>
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- IP: SOCS3
  - WB: pY
  - pY-SOCS3
  - WB: SOCS3
  - SOCS3 (IL-6 induced)

**NIH-3T3 Fibroblasts**

1st stimulation: IL-6/sIL-6R
2nd stimulation: PDGF

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<td>2nd stimulation: PDGF</td>
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- IP: SOCS3
  - WB: pY
  - pY-SOCS3
  - WB: SOCS3
  - SOCS3 (IL-6 induced)
Figure 8

C

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1st stimulation: IL-6/sIL-6R

2nd stimulation: PDGF

WB: pY

pY-SOCS3

WB: SOCS3

SOCS3 (IL-6 induced)

D

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3T3 SOCS3 WT

3T3 SOCS3 FF

WB: pY

pY-SOCS3

SOCS3-IP

WB: SOCS3

SOCS3

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