

Functional Cross-modulation between SOCS Proteins Can Stimulate Cytokine Signaling*

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SOCS (suppressors of cytokine signaling) proteins are negative regulators of cytokine signaling that function primarily at the receptor level. Remarkably, *in vitro* and *in vivo* observations revealed both inhibitory and stimulatory effects of SOCS2 on growth hormone signaling, suggesting an additional regulatory level. In this study, we examined the possibility of direct cross-modulation between SOCS proteins and found that SOCS2 could interfere with the inhibitory actions of other SOCS proteins in growth hormone, interferon, and leptin signaling. This SOCS2 effect was SOCS box-dependent, required recruitment of the elongin BC complex, and coincided with degradation of target SOCS proteins. Detailed mammalian protein-protein interaction trap (MAPPIT) analysis indicated that SOCS2 can interact with all members of the SOCS family. SOCS2 may thus function as a molecular bridge between a ubiquitin-protein isopeptide ligase complex and SOCS proteins, targeting them for proteasomal turnover. We furthermore extended these observations to SOCS6 and SOCS7. Our findings point to a unique regulatory role for SOCS2, SOCS6, and SOCS7 within the SOCS family and provide an explanation for the unexpected phenotypes observed in SOCS2 and SOCS6 transgenic mice.

Cytokine signaling is typically a transient event, implying rapid and finely tuned attenuation. Receptor binding leads to rapid activation of receptor-associated members of the JAK³ family. Subsequent phosphorylation of tyrosine residues in the receptor tails enables recruitment of downstream signaling molecules whereby STATs play a prominent role. Activated STATs translocate to the nucleus, where they control cytokine-regulated gene transcription. Negative control occurs at many

levels and involves receptor down-regulation, protein-tyrosine phosphatases, protein inhibitors of activated STATs, and members of the SOCS (suppressors of cytokine signaling) protein family.

The SOCS family consists of eight different members (SOCS1–7 and CIS (cytokine-inducible SH2 domain-containing protein)) characterized by conserved structural features. All SOCS proteins consist of a central SH2 domain flanked by a variable N-terminal region and a conserved C-terminal SOCS box (1, 2). The SH2 domain can inhibit STAT activation by direct competition for the phosphorylated receptor recruitment sites (3–8). SOCS1 and SOCS3 carry an additional kinase inhibitory region (KIR) in their N-terminal domains that acts as a pseudosubstrate for the JAK kinase, thereby blocking signaling (5). The SOCS box was shown to act as an interaction domain for the elongin BC complex (9, 10), which, in turn, is a component of an ubiquitin-protein isopeptide ligase (E3) complex (11). This way, the SOCS box can control protein turnover by marking target proteins for proteasomal degradation (12). However, the significance of the interaction between SOCS proteins and the elongin BC complex is not totally clarified, as some reports propose that elongin association targets SOCS molecules for proteasomal degradation (10, 12–15), whereas other data suggest that elongin BC binding stabilizes SOCS protein expression (9, 16, 17).

Deletion studies of SOCS genes in mice have underscored their importance in specific restricted cytokine signaling pathways, *e.g.* SOCS1-deficient mice suffer from deregulated interferon (IFN)- γ signaling characterized by malfunctioning of the immune system at several levels (18–20), and SOCS3 haploinsufficient mice or mice with a specific deletion of SOCS3 in hypothalamic neurons show augmented central leptin sensitivity (21, 22), suggesting a key role for SOCS3 in leptin resistance. A special case concerns SOCS2, which can have opposing effects on growth hormone (GH) signaling: SOCS2 knock-out mice exhibit an overgrowth phenotype because of prolonged GH-dependent STAT5 activity (23, 24), and paradoxically, overexpression of SOCS2 in a transgenic mouse model also leads to gigantism (25). This dual effect of SOCS2 is also observed *in vitro*, where low SOCS2 doses moderately inhibit GH signaling, and higher levels positively regulate signaling (25–27). A similar phenomenon is observed for the effect of SOCS2 on prolactin (PRL) (28) and interleukin-3 (29) signaling. The role of SOCS2 in regulating cytokine-induced signals is obviously complex because increasing SOCS2 levels can over-

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[§] The abbreviations used are: JAK, Janus kinase; STATs, signal transducers and activators of transcription; SH2, Src homology 2; E3, ubiquitin-protein isopeptide ligase; IFN, interferon; GH, growth hormone; PRL, prolactin; GHR, growth hormone receptor; LR, leptin receptor; EpoR, erythropoietin receptor; SEAP, secreted alkaline phosphatase; MAPPIT, mammalian protein-protein interaction trap.

come the negative effect of SOCS1 on GH receptor (GHR) and PRL signaling and can partially restore the SOCS3 down-regulated PRL function (26, 28, 30). Of note, SOCS6 overexpression also confers an enhanced phenotype because SOCS6 transgenic mice display increased insulin sensitivity and enhanced glucose metabolism (31).

GH, PRL, IFN, and others induce SOCS2 expression (27, 28, 32). Unlike SOCS1 and SOCS3 expression, which is typically induced in a rapid and transient manner, SOCS2 expression usually occurs later after cytokine stimulation and is more prolonged (28, 32). Consequently, it is tempting to speculate that SOCS2 may be involved in restoring cellular sensitivity by overcoming the inhibitory effect of other SOCS proteins. However, to date, no report concerning the precise molecular mechanism of action of SOCS2 in signal enhancement of GH response has been published.

This study was conducted to clarify the stimulatory effect of SOCS2 observed in GH signaling. We demonstrate that SOCS2 can interfere with the negative regulatory effects of SOCS1 and SOCS3 via direct interaction. This effect requires the C-terminal SOCS box of the targeted SOCS protein as well as the elongin BC-binding motif in the SOCS2 SOCS box, supporting proteasomal degradation of the targeted SOCS proteins. We also show that this inter-SOCS cross-modulation can be extended to other cytokine receptor systems and to other members of the SOCS protein family.

EXPERIMENTAL PROCEDURES

Constructs—All constructs used in this study were generated by standard PCR- or restriction-based cloning procedures and are represented in Table 1. The pEF-FLAG-I/mSOCS1, pEF-FLAG-I/mCIS, and pEF-FLAG-I/mSOCS2 constructs were kindly provided by Dr. R. Starr. pMET7-mLR (mouse leptin receptor (LR) long form) was a gift from Dr. L. Tartaglia, and the pcb6-rbGHR vector was a gift from Dr. G. Strous. The mouse thymus cDNA was kindly provided by Dr. P. Brouckaert. The pMET7-FLAG rat SOCS3 expression vector was described elsewhere (33). The pMET7-FLAG-CIS, pMET7-Etag-CIS, and pMET7-FLAG rat SOCS2 expression vectors have been described previously (34). Generation of the chimeric bait receptors containing the extracellular part of the erythropoietin receptor (EpoR) and the transmembrane and intracellular parts of the LR, such as pCEL, was described elsewhere (35, 36). Generation of the prey constructs pMG2-CIS and pMG2-SOCS2, both containing part of the gp130 chain (amino acids 905–918) in duplicate, was as described (37). The EpoR Tyr⁴⁰² bait and pMET7-SVT (SV40 large T antigen) expression vectors were obtained as described previously (36).

Cell Culture, Transfection Procedures, and Reagents—HEK293-T, 3T3-F442A, and N38 cells were cultured in a 10% CO₂ humidified atmosphere at 37 °C and grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum (Cambrex Corp.). For transfection experiments, cells were freshly seeded in 6-well plates. HEK293-T cells were transfected overnight with ~2.5 µg of plasmid DNA using a standard calcium phosphate precipitation procedure. The pMET7-SVT construct was used to normalize for the amount of transfected DNA and load of the transcriptional and translational machin-

ery. N38 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's guidelines. One day after transfection, cells were washed with phosphate-buffered saline without calcium, magnesium, and sodium bicarbonate and cultured until used. Recombinant mouse leptin and human erythropoietin were purchased from R&D Systems. Human GH was purchased from ImmunoTools, and human IFN-β was generated in the laboratory.

Luciferase and Secreted Alkaline Phosphatase (SEAP) Assays—For a typical luciferase experiment, HEK293-T or N38 cells were transfected with the desired constructs together with a luciferase reporter gene. For STAT5-dependent luciferase assays, we used a STAT5-responsive β-casein-derived luciferase reporter plasmid (38). For STAT3-dependent luciferase experiments, we used the pXP2d2-rPAP1-luciferase reporter, originating from the rat pancreatitis-associated protein 1 promoter, as described previously (36). 24 h after transfection, cells were left untreated or were stimulated with ligand. After another 24 h, luciferase activity from triplicate samples was measured by chemiluminescence in a TopCount luminometer (Canberra Packard). For IFN-stimulated gene factor 3-dependent SEAP assays, we used the 6-16 SEAP reporter construct, which was constructed as described previously (39). The amount of SEAP was determined with a Phospha-Light kit (Tropix, Inc., Bedford, MA) using disodium 3-(4-methoxy-3,2'-(5'-chloro)tricyclone[3.3.1.1]decan-4-yl)phenyl phosphate as the luminogen substrate. Assays were performed in a 96-well microtiter plate following the manufacturer's guidelines. Cells were lysed in 1% Triton X-100 and 20 mM Tris (pH 7.4), and alkaline phosphatase activity in triplicate samples was measured by chemiluminescence in the TopCount luminometer.

Western Blot Analysis and Co-immunoprecipitation—Transfected HEK293-T or N38 cells were lysed in modified radioimmune precipitation assay buffer (200 mM NaCl, 50 mM Tris-HCl (pH 8), 0.05% SDS, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM Na₃VO₄, 1 mM NaF, 20 mM β-glycerophosphate, and CompleteTM protease inhibitor mixture (Roche Applied Science)). 5× loading buffer (156 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue sodium salt, and 5% β-mercaptoethanol) was added to the cell lysates, which were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Blotting efficiency was checked by Ponceau S staining (Sigma). Blocking, washing, and incubation with antibodies were carried out in Tris-buffered saline supplemented with 5% dried skimmed milk and 0.1% Tween 20. FLAG-tagged (corresponding to the peptide tag DYKDDDDK) and E-tagged (corresponding to the peptide tag GAPVPYPDPLEPR) proteins were revealed using anti-FLAG monoclonal antibody M2 (Sigma) and anti-E tag monoclonal antibody (Amersham Biosciences), respectively. Rabbit anti-SOCS2 polyclonal antibody was a gift from Dr. J. Johnston, and anti-mouse β-actin antibody was supplied by Sigma. Immunoblots were then revealed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences) and SuperSignal West Pico chemiluminescent substrate (Pierce). For co-immunoprecipitation experiments, ~2 × 10⁶ HEK293-T cells were transfected

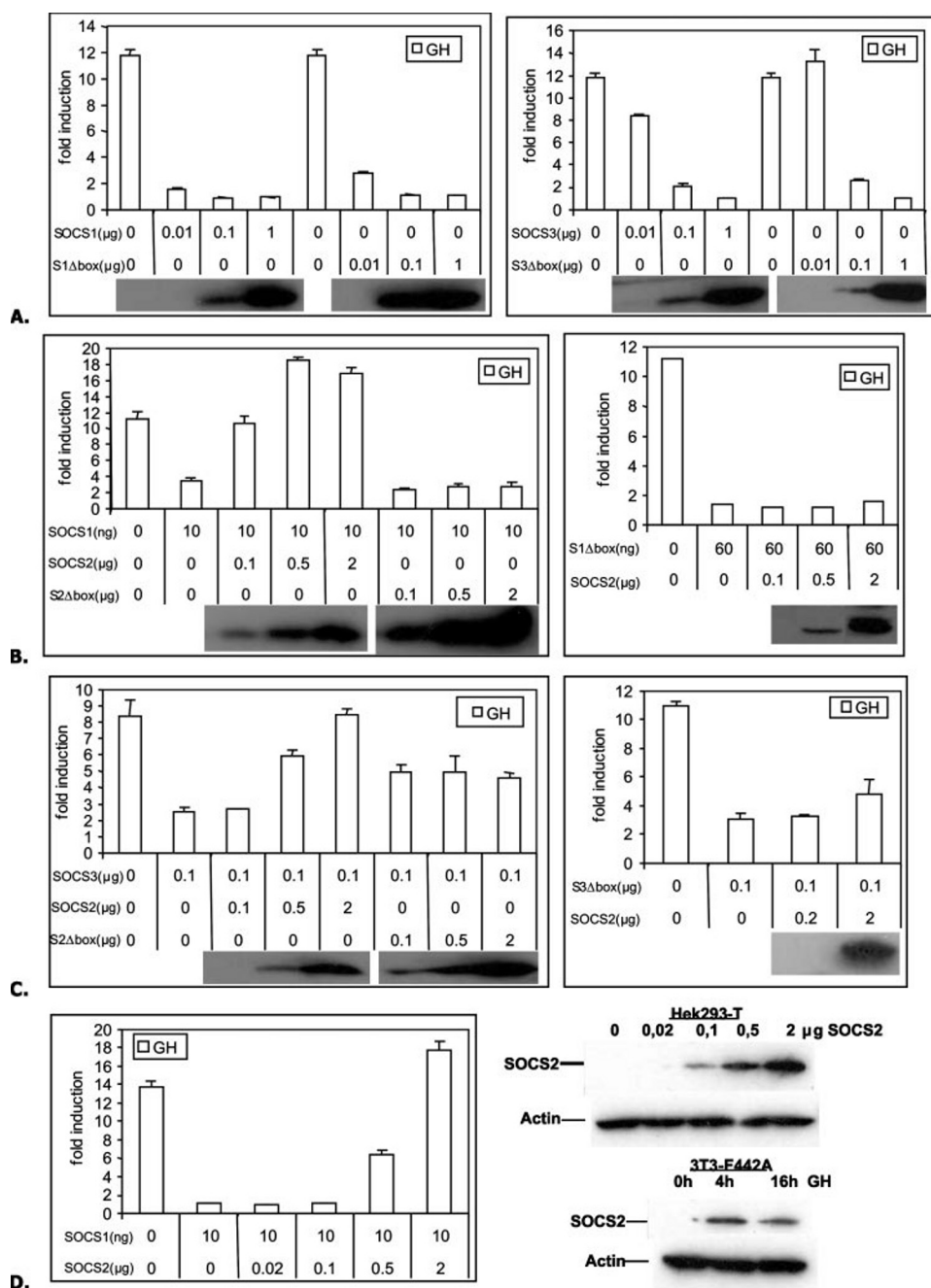


FIGURE 1. Essential role for the SOCS box in interference of SOCS2 with SOCS1 and SOCS3 inhibition of GH signaling. HEK293-T cells were transfected with a rabbit GHR expression vector (40 ng) and a β -casein-derived luciferase reporter gene (200 ng). 24 h after transfection, the cells were deprived of serum and then treated with human GH (200 ng/ml) for 15 h before the luciferase activity from the β -casein reporter gene was measured. Luciferase measurements were performed in triplicate. -Fold induction represents the ratio of luciferase activity determined in the presence or absence of ligand. **A**, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids (SOCS1 Δ box (S1 Δ box) and SOCS3 Δ box (S3 Δ box)) were cotransfected at a range of concentrations to analyze the inhibitory effect on GH signaling. A sample of lysate from each group was Western-blotted and probed with anti-FLAG antibody. **B**, SOCS1 (10 ng) or SOCS1 Δ box (60 ng) was cotransfected with SOCS2 or SOCS2 Δ box (S2 Δ box) at increasing concentrations. Expression of the E-tagged SOCS2 and SOCS2 Δ box proteins in the same transfected cells was verified on lysates using anti-E tag antibody. **C**, SOCS3 (100 ng) or SOCS3 Δ box (100 ng) was cotransfected with SOCS2 or SOCS2 Δ box at increasing concentrations. Expression of the E-tagged SOCS2 and SOCS2 Δ box proteins in the same transfected cells was verified on lysates using anti-E tag antibody. **D**, shown is a comparison of the ectopic and endogenous expression levels of mouse SOCS2 in HEK293-T and 3T3-F442A cells, respectively. SOCS1 (10 ng) was cotransfected with SOCS2 at increasing concentrations in HEK293-T cells. Expression of the E-tagged SOCS2 proteins in the same transfected cells and of endogenous SOCS2 in the 3T3-F442A cells was verified on lysates using anti-SOCS2 antibody. 3T3-F442A cells were incubated in serum-free medium prior to stimulation with GH (200 ng/ml) for the indicated times. The levels of loaded protein were normalized by determining the protein concentrations with the Bradford method, and this was verified by Ponceau S staining. As an additional control, the blots were stripped and probed with anti- β -actin antibody to check for equal loading of lysates of the same cell type.

with FLAG- or E-tagged pMET7-SOCS expression vectors. Cleared lysates (modified radioimmune precipitation assay lysis buffer) were incubated with 4.0 μ g/ml mouse anti-FLAG or anti-E tag monoclonal antibody and protein G-Sepharose (Amersham Biosciences). After immunoprecipitation, SDS-PAGE, and Western blotting, interactions were detected using anti-FLAG or anti-E tag antibody as described above.

RESULTS

Essential Role for the SOCS2 SOCS Box in Antagonizing SOCS1 and SOCS3 Inhibition of Cytokine Signaling—SOCS2 exerts a dual action on GH and PRL signaling and impairs the inhibitory effect of other SOCS proteins (26, 30). To gain more detailed insight in the underlying mechanism, we first analyzed the role of the different SOCS protein subdomains. The effect of constitutive expression of SOCS proteins on GH signaling was investigated in HEK293-T cells using the STAT5-responsive β -casein-derived luciferase reporter. Fig. 1A shows dose-response curves demonstrating complete inhibition of GH signaling by SOCS1 and SOCS3. GH-inducible activity was fully inhibited by low concentrations of either SOCS1 or SOCS3, *i.e.* concentrations below the level of antibody detection as judged by Western blot analysis of the FLAG-tagged SOCS constructs using anti-FLAG antibody. After removal of the SOCS boxes of SOCS1 (SOCS1 Δ box) and SOCS3 (SOCS3 Δ box), inhibition was slightly reduced but not abolished in this assay system. Coexpression of SOCS2 completely suppressed the SOCS1- and SOCS3-dependent inhibition of GH signaling (Fig. 1, B and C), whereby SOCS1 inhibition appeared to be more sensitive to the counteracting effect of SOCS2 compared with SOCS3. Of note, the amounts of SOCS1 used in this experiment could not be visualized by Western blot analysis, indicating that the working concentrations

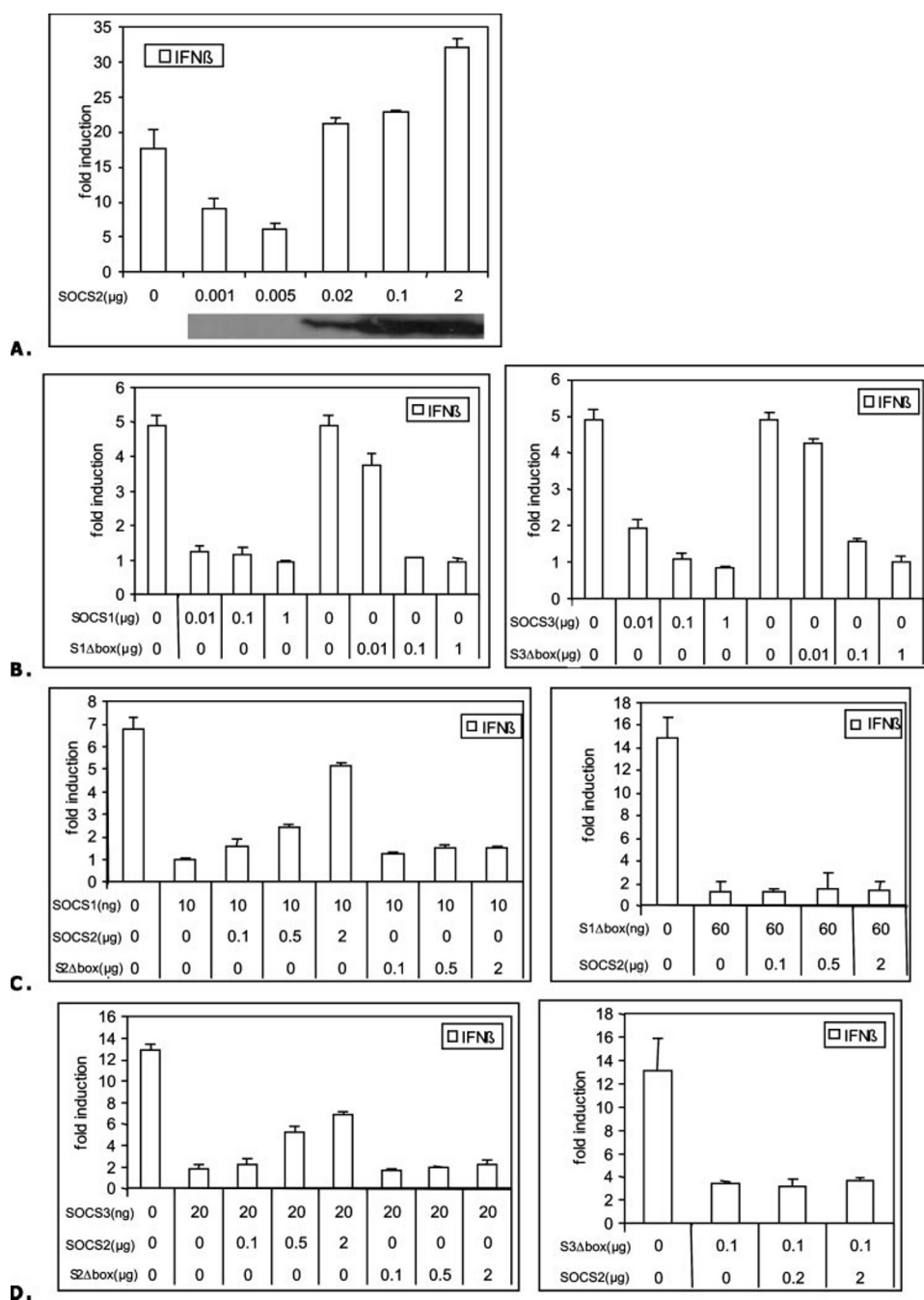


FIGURE 2. Essential role for the SOCS box in interference of SOCS2 with SOCS1 and SOCS3 inhibition of IFN signaling. SEAP activity was assayed in HEK293-T cells transfected without SOCS or with SOCS1–3 expression vectors and the IFN-responsive 6-16 SEAP reporter (200 ng) at several ratios. After 24 h, transfected cells were stimulated with human IFN- β (100 pM); and after 48 h, the SEAP activity in the 6-16 reporter gene was measured. SEAP measurements were performed in triplicate. -Fold induction represents the ratio of SEAP activity determined in the presence or absence of ligand. **A**, SOCS2 can act as a dual effector of IFN type I signaling as assessed by transfection with SOCS2 at a range of concentrations or with empty vector in an appropriate amount in HEK293-T cells. Expression of the E-tagged SOCS2 protein in the same transfected cells was verified on lysates using anti-E tag antibody. **B**, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids were transfected at a range of concentrations to analyze the inhibitory effect on interferon type I signaling. **C**, SOCS1 (10 ng) or SOCS1Δbox (S1Δbox; 60 ng) was cotransfected with SOCS2 or SOCS2Δbox (S2Δbox) at increasing concentrations. **D**, SOCS3 (20 ng) or SOCS3Δbox (S3Δbox; 100 ng) was cotransfected with SOCS2 or SOCS2Δbox at increasing concentrations.

were approaching the physiological concentrations of this SOCS protein. The SOCS2 amounts were also not supra-physiological, as the SOCS2 concentration at which a cross-regulatory effect was observed was comparable with the

endogenous level of SOCS2 in the GH-responsive mouse 3T3-F442A preadipocyte cell line (Fig. 1D). This suppressive effect on SOCS molecules was specific for SOCS2, as coexpression of CIS did not interfere with SOCS1- or SOCS3-mediated inhibition (data not shown). Strikingly, this effect of SOCS2 depended strictly on the presence of its SOCS box. Of note, the deletion of the SOCS box led to enhanced expression in the case of SOCS2. This effect was also observed, albeit to a lesser extent, with SOCS1, but was not observed for SOCS3.

We next evaluated whether we could extrapolate this SOCS2 regulation to other receptor systems. SOCS1 and SOCS3 have been implicated as potent inhibitors of IFN type I receptor signaling (40, 41); however, the role of SOCS2 is less well elucidated. We monitored IFN- β signaling in HEK293-T cells using the IFN-sensitive 6-16 SEAP type I reporter gene and evaluated the effect of expression of various combinations of (mutant) SOCS proteins as described above. We found that expression of SOCS2 at increasing concentrations resulted in a clear dual effect on IFN signaling (Fig. 2A): at low concentrations, SOCS2 suppressed IFN signaling, but at higher concentrations, SOCS2 led to complete restoration and even enhancement of the responsiveness of the 6-16 reporter to IFN- β , suggesting a negative effect of SOCS2 on endogenous SOCS proteins. Quite similar to what we observed with GH, expression of SOCS1, SOCS3, or their mutants lacking the SOCS box inhibited IFN- β signaling (Fig. 2B). Again, analogous to the observations made for GH, SOCS1- and SOCS3-mediated inhibition of IFN- β signaling was completely neutralized by coexpression of SOCS2, and the SOCS boxes of SOCS1 or SOCS3 and of SOCS2

were strictly required for the full effect (Fig. 2, C and D).

We finally extended these analyses of SOCS modulation also to LR signaling. Again, quite similar to the previous observations, expression of SOCS1, SOCS3, or their SOCS box deletion

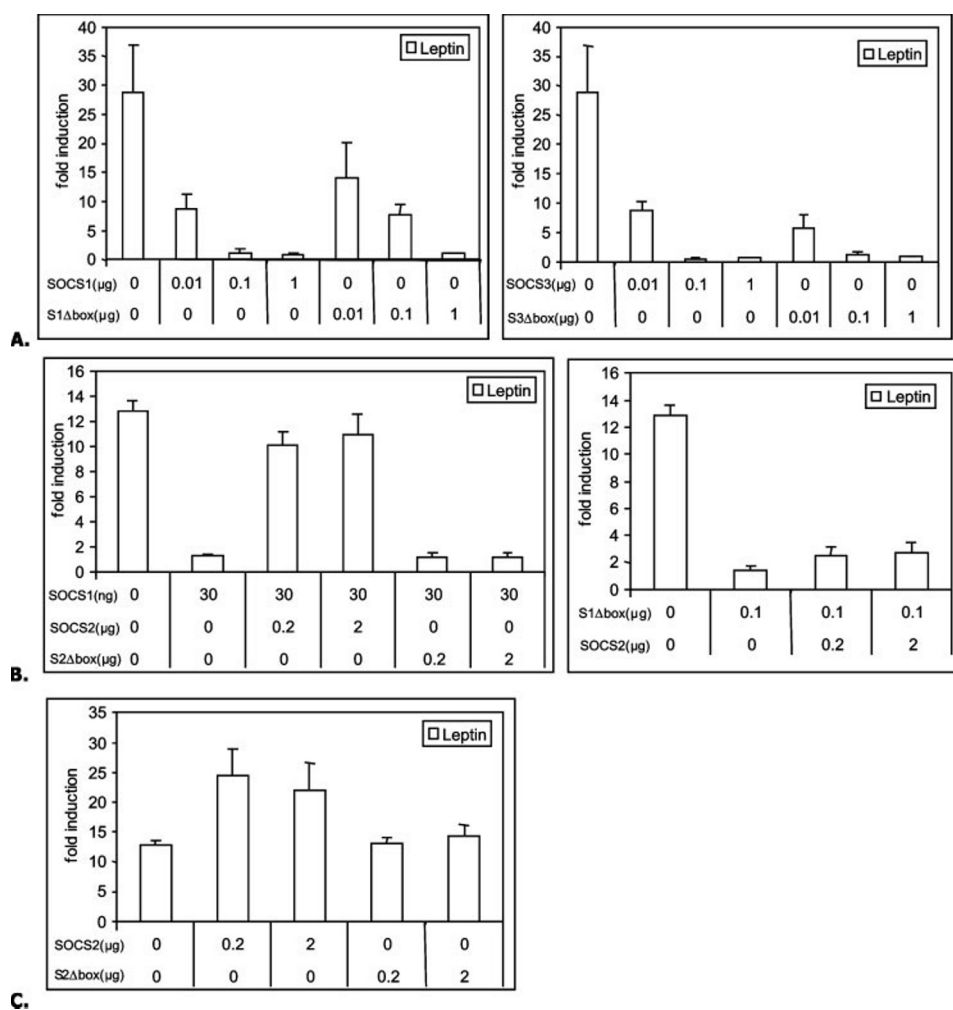


FIGURE 3. SOCS2 displays a SOCS box-dependent stimulatory effect on leptin signaling in N38 cells. Mouse N38 hypothalamic cells were transiently cotransfected with a mouse LR expression vector (250 ng) and the pXP2d2-rPAP1-luciferase reporter (1 μg). The transfected cells were either left untreated or stimulated for 24 h with leptin (100 ng/ml). Luciferase measurements were performed in triplicate. Data are expressed as the leptin-stimulated/non-stimulated ratio. *A*, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids were cotransfected at a range of concentrations to analyze the inhibitory effect on leptin signaling. *B*, SOCS1 (30 ng) or SOCS1Δbox (S1Δbox; 100 ng) was cotransfected with SOCS2 or SOCS2Δbox (S2Δbox) at increasing concentrations. *C*, SOCS2 or the SOCS2Δbox mutant plasmid was transfected at a range of concentrations to analyze the stimulatory effect on leptin signaling.

mutants blocked induction of leptin-mediated activation of a STAT3-responsive rat pancreatitis-associated protein 1 promoter-luciferase reporter in HEK293-T cells (data not shown). Coexpression of SOCS2 restored the SOCS-dependent signaling blockade. These effects were dependent on either SOCS box and were less pronounced for SOCS3-mediated LR inhibition than for SOCS1-mediated LR inhibition (data not shown). We further verified the cross-modulatory effects of SOCS2 in mouse N38 hypothalamic cells, which represent a physiological context for LR signaling. This N38 cell line responds to leptin stimulation and is a part of a collection of clonal neuronal cell lines recently isolated by Belsham *et al.* (42). Similar to what we observed in HEK293-T cells, expression of SOCS1, SOCS3, or their mutants lacking the SOCS box inhibited leptin signaling (Fig. 3A). Coexpression of SOCS2 with SOCS1 (Fig. 3B) or SOCS3 (data not shown) in the N38 cells led to recovery of the leptin-induced signaling in a SOCS box-dependent manner. Moreover, expression of SOCS2 alone clearly stimulated

the STAT3-dependent luciferase response (Fig. 3C), which can be explained by a negative effect of SOCS2 on the endogenous SOCS proteins. This effect was again lost with a SOCS2 mutant lacking the SOCS box. Together, these findings show that the cross-modulatory effect of SOCS2 on other SOCS proteins is not limited to the GH system and likely involves similar underlying mechanisms.

Recruitment of the Elongin BC Complex by SOCS2 Is Essential for Interference with Other SOCS Proteins—Sequence alignments of SOCS box-containing proteins reveal a single conserved region with the consensus sequence (T/S)(L/M)XXX(C/S)-XXX(V/L/I) that defines an elongin BC complex-binding site or “BC box” (9, 10, 43). We generated a SOCS2 mutant, SOCS2(LC-PF), containing point mutations in the BC box of SOCS2 that abrogate elongin BC recruitment (9). In another SOCS2 derivative, SOCS2(LC-QQ), both residues were mutated to glutamine to minimize structural alterations of the protein. As shown in Fig. 4, this SOCS2(LC-QQ) mutant completely lost its capacity to interfere with SOCS1 and SOCS3 antagonism in GH and IFN signaling in HEK293-T cells and with leptin signaling in N38 cells. Similar findings were made with the SOCS2(LC-PF) mutant (data not shown). This indicates that functional recruitment of the

elongin BC complex is a prerequisite for the negative regulation by SOCS2 of other SOCS proteins.

SOCS2 Interacts with Other Members of the SOCS Family—We next used mammalian protein-protein interaction trap (MAPPIT), a strategy designed to analyze protein-protein interactions in intact mammalian cells (36), to investigate whether SOCS2 exerts its cross-modulatory function via direct binding to other SOCS proteins. In MAPPIT, a bait protein is C-terminally linked to a chimeric receptor consisting of the extracellular region of the EpoR linked to the transmembrane and the intracellular part of a signaling-deficient LR. The use of a triple Tyr-to-Phe mutant LR (further referred to as LR-F3) knocks out STAT3 activation and offers the added advantage that negative feedback mechanisms are inoperative, implying enhanced signaling.

MAPPIT prey constructs are composed of a prey protein fused to a part of the gp130 chain carrying four STAT3 recruitment sites. Coexpression of interacting bait and prey

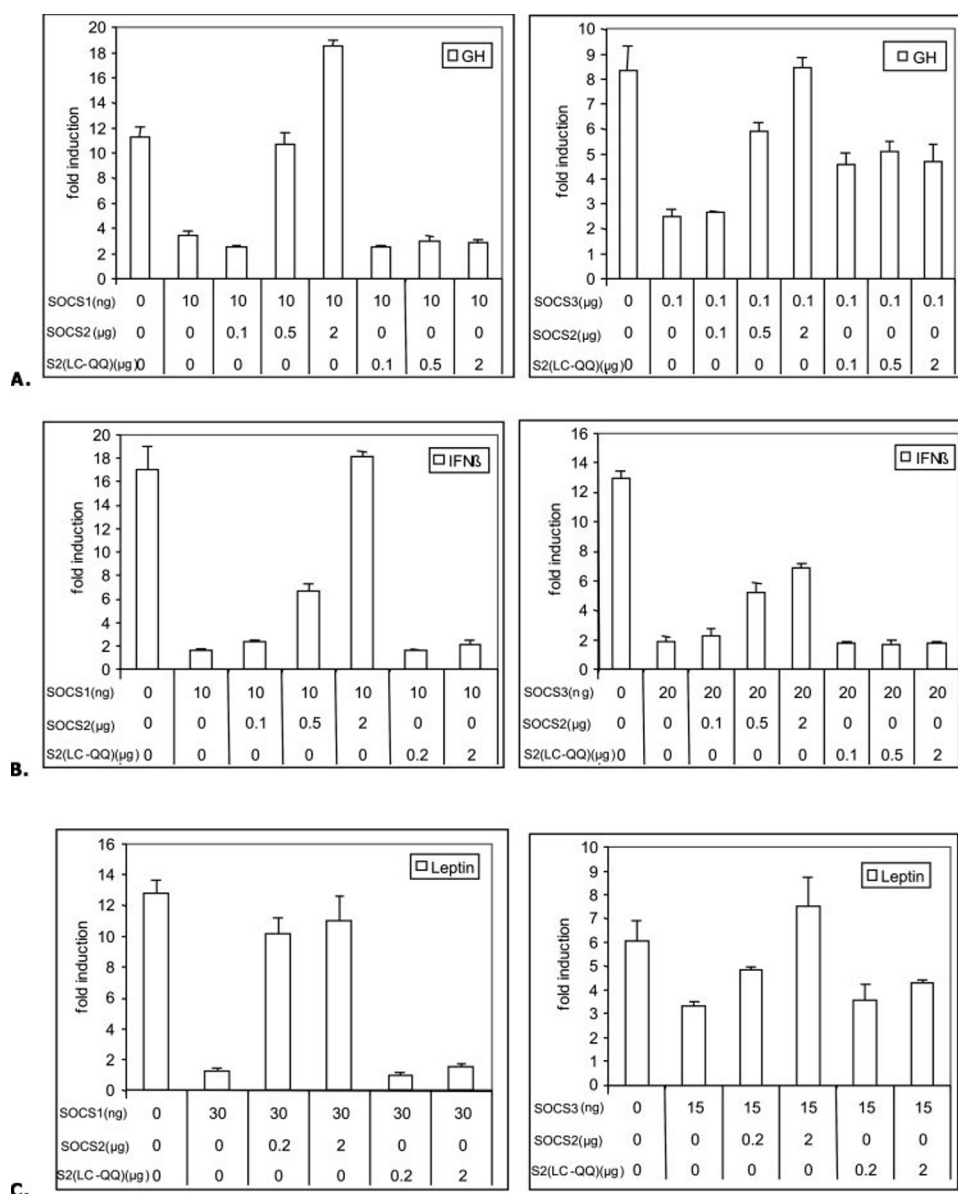


FIGURE 4. The negative effect of SOCS2 on other SOCS proteins is dependent on recruitment of the elongin BC complex. HEK293-T or N38 cells were transiently transfected with plasmids encoding SOCS1 or SOCS3 at fixed amounts and with wild-type SOCS2 or SOCS2ΔBC box(LC-QQ) (S2(LC-QQ)) at increasing concentrations. The transfected cells were either left untreated or were stimulated with human GH (200 ng/ml), IFN-β (100 pM), or leptin (100 ng/ml). Luciferase and SEAP measurements were performed in triplicate. Data are expressed as -fold induction (stimulated/non-stimulated). **A**, HEK293-T cells were transfected with 10 ng of SOCS1, 100 ng of SOCS3, and increasing concentrations of SOCS2 derivatives. GH signaling was assayed as described in the legend to Fig. 1. **B**, HEK293-T cells were transfected with 10 ng of SOCS1, 20 ng of SOCS3, and increasing concentrations of SOCS2 derivatives. IFN signaling was assayed as described in the legend to Fig. 2. **C**, N38 cells were transfected with 30 ng of SOCS1, 15 ng of SOCS3, and increasing concentrations of SOCS2 derivatives. Leptin signaling was assayed as described in the legend to Fig. 3.

leads to functional complementation of STAT3 activity and induction of the STAT3-responsive rat pancreatitis-associated protein 1 promoter-luciferase reporter. MAPPIT permits the detection of both modification-independent and phosphorylation-dependent interactions in intact human cells. The MAPPIT configuration used in this study is shown in Fig. 5A.

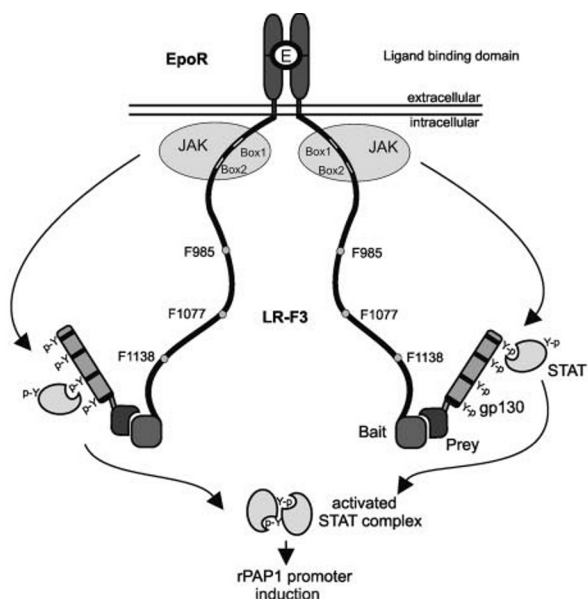
We have shown previously that SOCS2 directly interacts with CIS (34). This observation and the abovementioned findings on cross-regulation between SOCS2 and SOCS1 or SOCS3

prompted us to investigate whether SOCS2 can bind to these SOCS family members. HEK293-T cells were cotransfected with a bait plasmid encoding the SOCS boxes of SOCS1, SOCS2, SOCS3, or CIS combined with a plasmid encoding the SOCS2 or CIS prey and the STAT3-responsive luciferase reporter construct. We always used isolated SOCS boxes as bait proteins because, in the case of SOCS1 and SOCS3, the full-length baits interfered with the MAPPIT readout and therefore could not be investigated. Erythropoietin stimulation revealed clear interaction of SOCS2 with all baits examined. In contrast, the CIS prey failed to induce any reporter activity (Fig. 5B). The expression levels of the FLAG-tagged prey proteins were confirmed by immunoblotting using anti-FLAG antibody. In Fig. 5C, we show, using MAPPIT, that the SOCS2(LC-QQ) mutant lost its capacity to associate with an elongin B prey while maintaining its interaction with the CIS bait. A MAPPIT bait construct containing the Tyr⁴⁰² motif of the EpoR was used as a positive control, as this receptor motif directly interacts with SOCS2 (36). This association of SOCS2 with SOCS1–3 and CIS was confirmed by co-immunoprecipitation. We transiently cotransfected HEK293-T cells with a plasmid encoding E-tagged SOCS2 together with FLAG-tagged plasmids encoding SOCS1, SOCS2, and SOCS3, respectively, or we coexpressed FLAG-tagged SOCS2 with E-tagged SOCS1, SOCS2, and CIS. SOCS2 co-immunoprecipitated SOCS1–3 (Fig. 6A) and, in the case of CIS, both the 37- and 32-kDa forms that correspond to mono-

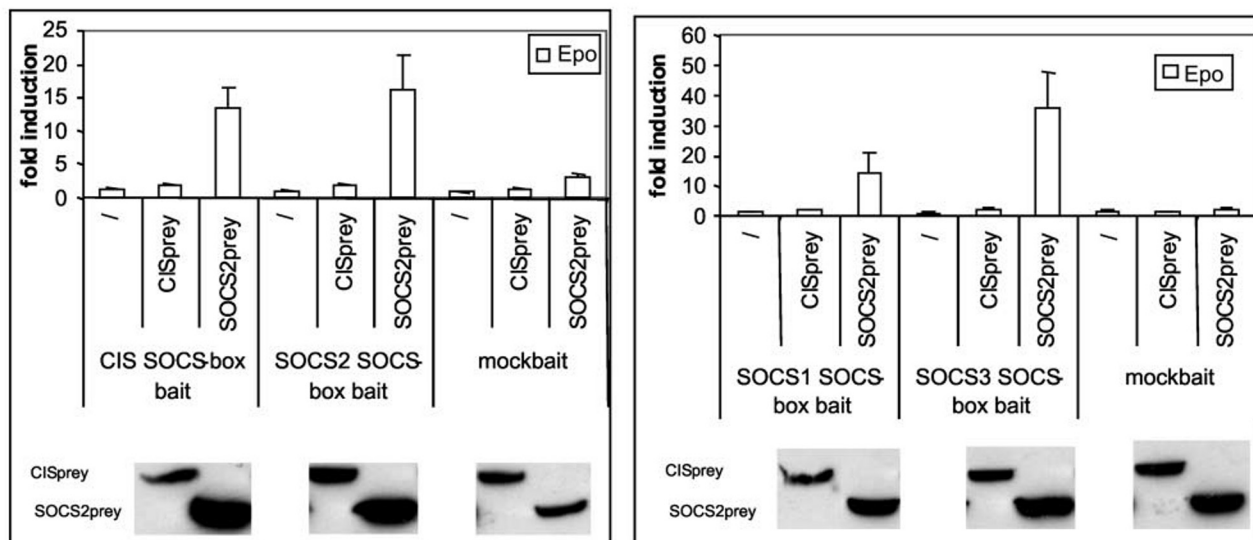
and non-ubiquitinated proteins, respectively (44). Observed interactions of SOCS2 with SOCS proteins in HEK293-T cells depended on proteasomal inhibition with the proteasomal inhibitor MG132 (20 μM) for 6 h and stimulation with IFN-β (100 pM) for 30 min. SOCS1 was still co-immunoprecipitated with the SOCS2Δbox and SOCS2(LC-QQ) mutants, indicating that deletion of the SOCS box or the BC box motif did not disrupt the capacity of SOCS2 to bind SOCS1 (Fig. 6B). Nevertheless, elimination of the SOCS box of SOCS2 weakened the interaction with SOCS1, suggesting a role for this domain in SOCS-SOCS interactions.

SOCS Cross-regulation

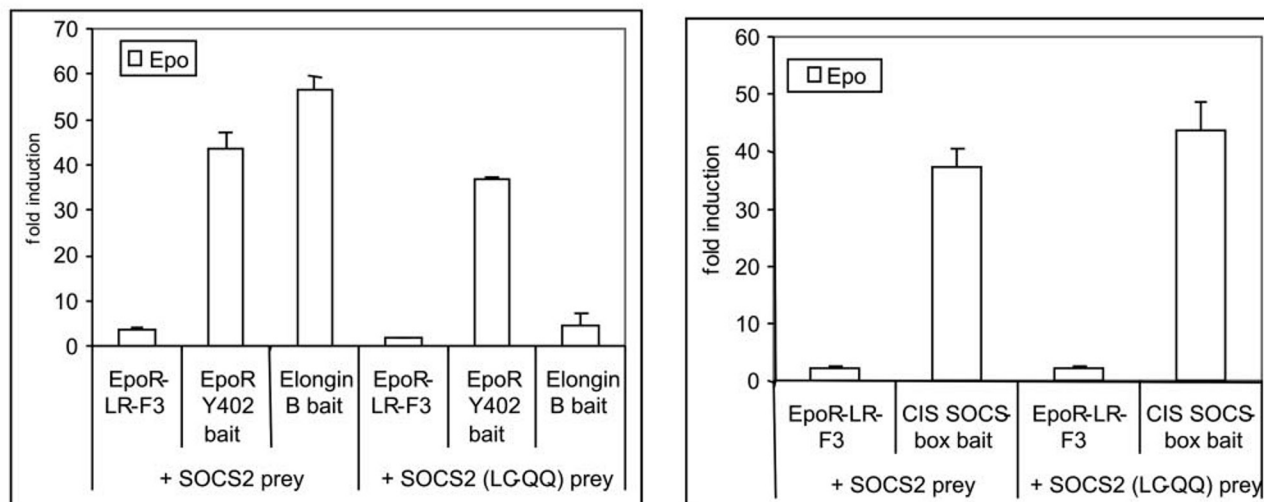
A.



B.



C.



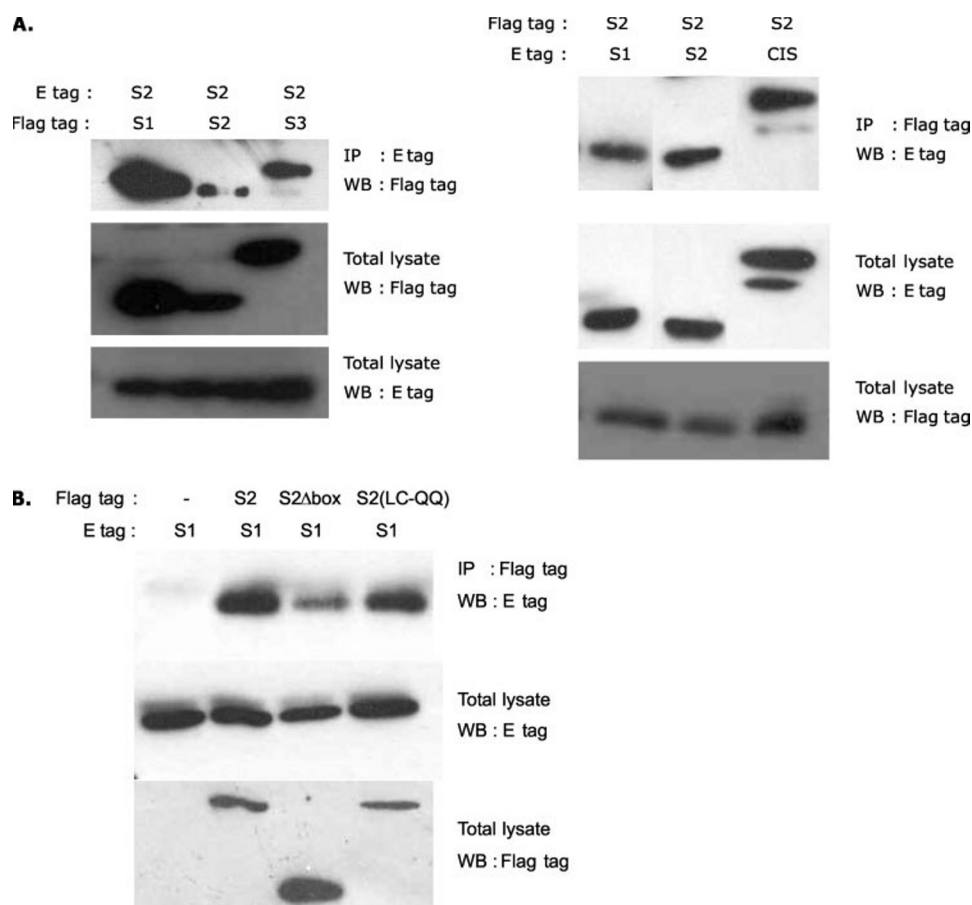


FIGURE 6. A, SOCS2 interactions demonstrated by co-immunoprecipitation experiments. Lysates from HEK293-T cells cotransfected with FLAG- or E-tagged SOCS1 (S1), SOCS2 (S2), SOCS3 (S3), and CIS were immunoprecipitated (IP) with anti-E tag or anti-FLAG antibody and Western-blotted (WB) with anti-FLAG or anti-E tag antibody (upper panels). The whole cell lysate was Western-blotted with anti-FLAG or anti-E tag antibody as a loading control (middle and lower panels). B, interaction analysis of SOCS1 and SOCS2 mutants. HEK293-T cells were transiently cotransfected with FLAG-tagged SOCS2, SOCS2 Δ box (S2 Δ box), SOCS2 Δ BC box(LC-QQ) (S2(LC-QQ)), or the appropriate amount of empty vector and E-tagged SOCS1. Cell lysates were immunoprecipitated with anti-FLAG antibody and subsequently immunoblotted with anti-E tag or anti-FLAG antibody.

SOCS2 Promotes Degradation of SOCS1—The dependence of the SOCS2 effect on an intact BC box suggests that SOCS2 can target SOCS proteins for proteasomal degradation. HEK293-T cells were transiently transfected with SOCS1 and increasing concentrations of SOCS2 and then treated with the protein synthesis inhibitor cycloheximide (20 μ M) for 6 h. Degradation of SOCS1 was observed when increasing concentrations of SOCS2 were coexpressed, whereas SOCS2(LC-QQ) had no effect (Fig. 7). This suggests a mechanism in which SOCS2 acts as an adaptor molecule between an E3 complex and SOCS proteins, targeting them for proteasomal turnover.

SOCS2 and Also SOCS6 and SOCS7 Interact with All Members of the SOCS Family—Interaction studies performed with the other SOCS proteins used as baits revealed that the SOCS2 prey can also interact with the SOCS boxes of SOCS4–7 (Fig. 8A). Using the same approach, we performed a matrix-type interaction analysis of SOCS proteins, and we found that SOCS6 and SOCS7 preys also interacted with the SOCS box baits of all members of the SOCS family (Fig. 8, B and C). Of note, SOCS2, SOCS6, and SOCS7 also displayed binding to themselves. The MAPPIT data with the CIS prey were included as a negative control, and functionality of this CIS prey was demonstrated using the interaction with the EpoR Tyr⁴⁰² motif as a control (Fig. 8D). The EpoR-LR-F3 bait provided a control for aspecific binding to the intracellular part of the LR and JAK2. The expression of the different bait constructs was verified by checking the interaction with the SH2 β prey, which binds the associated JAK of LR-F3 (data not shown). Possible complications that could arise from interference of the SOCS prey constructs with JAK activity or STAT recruitment and that could lead to false negative signals were considered and ruled out, as their coexpression with an established

MAPPIT interaction had no deleterious effect (data not shown). From these experiments, we concluded that SOCS2, SOCS6, and SOCS7 can interact with the SOCS boxes of all SOCS family members.

SOCS6 Is a Negative Regulator of Other SOCS Proteins—Subsequently, we investigated whether SOCS6 displays similar functional SOCS cross-modulation as SOCS2. We found that SOCS6 antagonized the inhibition of SOCS proteins in GH and IFN (Fig. 9, A and B) and leptin (data not shown) signaling in HEK293-T cells. In N38 cells, SOCS6 interfered with SOCS1 and SOCS3 inhibition of leptin signaling (Fig. 9C).

FIGURE 5. **MAPPIT analysis of SOCS interactions.** A, principle of MAPPIT. See “Results” for details. rPAP1, rat pancreatitis-associated protein 1. B, SOCS2 interacts with the SOCS boxes of SOCS1–3 and CIS. HEK293-T cells were transiently cotransfected with plasmids encoding bait variants of the SOCS boxes of several SOCS proteins or with a mock bait lacking the SOCS motif, the pMG2-SOCS2 or pMG2-CIS prey construct, and the pXP2d2-rPAP1-luciferase reporter. After transfection, cells were either left untreated or were stimulated with erythropoietin (Epo) for 24 h. Luciferase activities were measured in triplicate. -Fold induction represents the ratio of luciferase activity determined in the presence or absence of ligand. Expression of the FLAG-tagged fusion prey proteins in the same transfected cells was verified on lysates using anti-FLAG antibody. C, the SOCS2(LC-QQ) mutant does not bind elongin B, whereas the interaction with CIS is preserved. HEK293-T cells were transiently cotransfected with plasmids encoding the chimeric EpoR-LR-F3 construct as a negative control, the EpoR Tyr⁴⁰² bait as a positive control for SOCS2, the elongin B bait, or the CIS SOCS box bait and with the pMG2-SOCS2 or pMG2-SOCS2(LC-QQ) prey construct combined with pXP2d2-rPAP1-luciferase. The transfected cells were either stimulated for 24 h with erythropoietin or were left untreated. Luciferase measurements were performed in triplicate. Data are expressed as -fold induction (stimulated/non-stimulated).

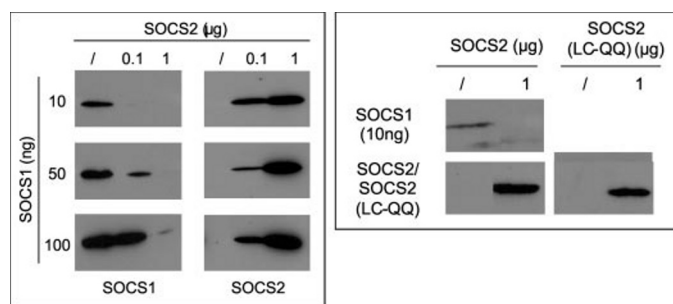


FIGURE 7. **Coexpression of SOCS2 accelerates SOCS1 degradation.** Increasing concentrations of E-tagged SOCS2 or SOCS2(LC-QQ) were transiently coexpressed in HEK293-T cells with FLAG-tagged SOCS1. Cells were treated with cycloheximide (20 μ M) for up to 6 h. The lysates were blotted for SOCS1 with anti-FLAG antibody and for SOCS2 with anti-E tag antibody.

Like other SOCS molecules, SOCS6 was shown to bind to elongins B and C in a SOCS box-dependent manner (45). Analogous to SOCS2, disruption of elongin BC binding in SOCS6 yielded a mutant that was not able to interfere with the inhibitory effect of other SOCS proteins (Fig. 9). Also, wild-type SOCS6, but not the Δ BC box mutant, reduced SOCS1 expression in a dose-dependent manner, indicating that SOCS6 mediated the observed inhibition by accelerating turnover of other SOCS proteins (Fig. 10). Taken together, our data suggest that SOCS6 can negatively regulate SOCS function in a way very similar to SOCS2.

DISCUSSION

Protein degradation by the ubiquitin-proteasome pathway plays an essential role in controlling the abundance of regulatory molecules. Key to this is the sequential action of three protein sets: ubiquitin-activating enzymes, ubiquitin carrier enzymes, and a large set of E3 enzymes, whereby the latter define substrate specificity. The SCF (Skp1-Cul1-F-box) E3 complex is composed of the Cul1 scaffold protein, which binds the Roc1/Rbx1 RING domain protein and the ubiquitin carrier enzyme and which recruits, via the Skp1 linker protein, F-box proteins, which in turn bind substrates for ubiquitination. This same architecture is also found in other SCF-like complexes, including those based on the Cul2-von Hippel-Lindau and Cul5-SOCS box adaptor proteins, whereby elongins B and C and SOCS or von Hippel-Lindau proteins fulfil the role of the Skp1 and F-box protein moieties, respectively (1). Evidence that SOCS proteins can mediate proteasomal turnover of target molecules is accumulating. Examples include the GHR and EpoR (44, 46), JAK2 (13), the Rac guanine nucleotide exchange factor Vav (47), Ras GTPase-activating protein (17), and insulin receptor substrates 1 and 2 (48). Of note, SOCS proteins themselves can be targeted for ubiquitination and proteasomal degradation, although contradictory reports exist regarding the effect of elongin BC interaction on the protein stability of SOCS1, SOCS3 and CIS. Some data suggest that elongin BC association targets SOCS proteins for degradation by the proteasome, as has been demonstrated for CIS (44, 49), SOCS1 (10, 50), and SOCS3 (10, 51). In contrast, there is also evidence that elongin BC interaction can stabilize SOCS1 (9, 16, 52) and SOCS3 (17) and that disruption of this interaction leads to proteasome-mediated degradation of these SOCS proteins.

SOCS2 undeniably plays a role as a negative regulator of GH signaling *in vivo* and *in vitro* (43), but can also enhance GH signaling when expressed at higher concentrations (25, 26). It binds to the GHR at multiple sites, some of which could also function as recruitment sites for negative regulators such as SHP-2 (53) and SOCS3 (54, 55). Such competition between SOCS2 and potentially more potent negative regulators was put forward as a potential explanation for the dual effect of SOCS2 (25). However, little direct evidence was reported in support of such a model, and recently, Greenhalgh *et al.* (43) showed that SOCS2 binds the GHR at Tyr⁴⁸⁷ and Tyr⁵⁹⁵, which are not usual immunoreceptor tyrosine-based inhibitory motifs, suggesting that competition of SOCS3 at these sites is not involved.

The key finding in this study is that a restricted set of SOCS proteins, including SOCS2, can bind to other members of the SOCS family, thus controlling their activity through proteasome-dependent degradation. We found that SOCS2 can restore and potentiate GH signaling by antagonizing SOCS1 and SOCS3 in a SOCS box-dependent manner. This effect is not limited to the GH system because we found similar effects on signaling via the endogenous IFN type I receptor and LR in HEK293-T and N38 cells, respectively.

SOCS2 mutants lacking the binding site for elongin BC completely lose their inhibitory potential, providing a strong argument for proteasomal degradation of the target SOCS proteins. Indeed, as observed for SOCS1, coexpression of SOCS2 leads to lowered expression levels of this target SOCS protein. The critical elongin BC dependence of the inhibitory effect by SOCS2 strongly argues that SOCS2 functions as part of an E3 complex. Alternatively, higher expression levels of SOCS2 may compete for recruitment of the elongin BC complex, indirectly leading to destabilization of other SOCS proteins lacking this complex (see above). However, SOCS1 and SOCS3 proteins lacking their entire SOCS boxes are still able, although to a lesser extent, to inhibit cytokine signaling, but are completely refractory to the SOCS2 effect, implying that SOCS2 binding is critical. Moreover, overexpression of CIS, which is equally well capable of sequestering elongin BC complexes, does not lead to any effect on other SOCS proteins. The SOCS box of the target SOCS protein appears to be involved in SOCS2 binding. Although our data support an involvement of the SOCS box in the interaction between the inhibitory SOCS and targeted SOCS proteins, the precise nature of this inter-SOCS interaction is still unclear and, given the MAPPIT configuration, may well depend on phosphorylation of critical tyrosine residues. Mutational analysis will be required to fully determine the binding modus between different SOCS proteins.

Evidence that SOCS2 can act as a regulator of turnover of other SOCS proteins was recently also reported by Tannahill *et al.* (29), who demonstrated SOCS2 regulation of the SOCS3-dependent inhibition of interleukin-2 and interleukin-3 signaling, and by Lavens *et al.* (34), who showed elongin BC-dependent interference of SOCS2 with binding of CIS to the LR at Tyr⁹⁸⁵. In line with such a regulatory role of SOCS proteins is the sequential induction pattern of different SOCS molecules. Unlike expression of SOCS1 and SOCS3, which is typically induced in a rapid and transient manner upon cytokine stimulation, expression of SOCS2 usually occurs late after cytokine

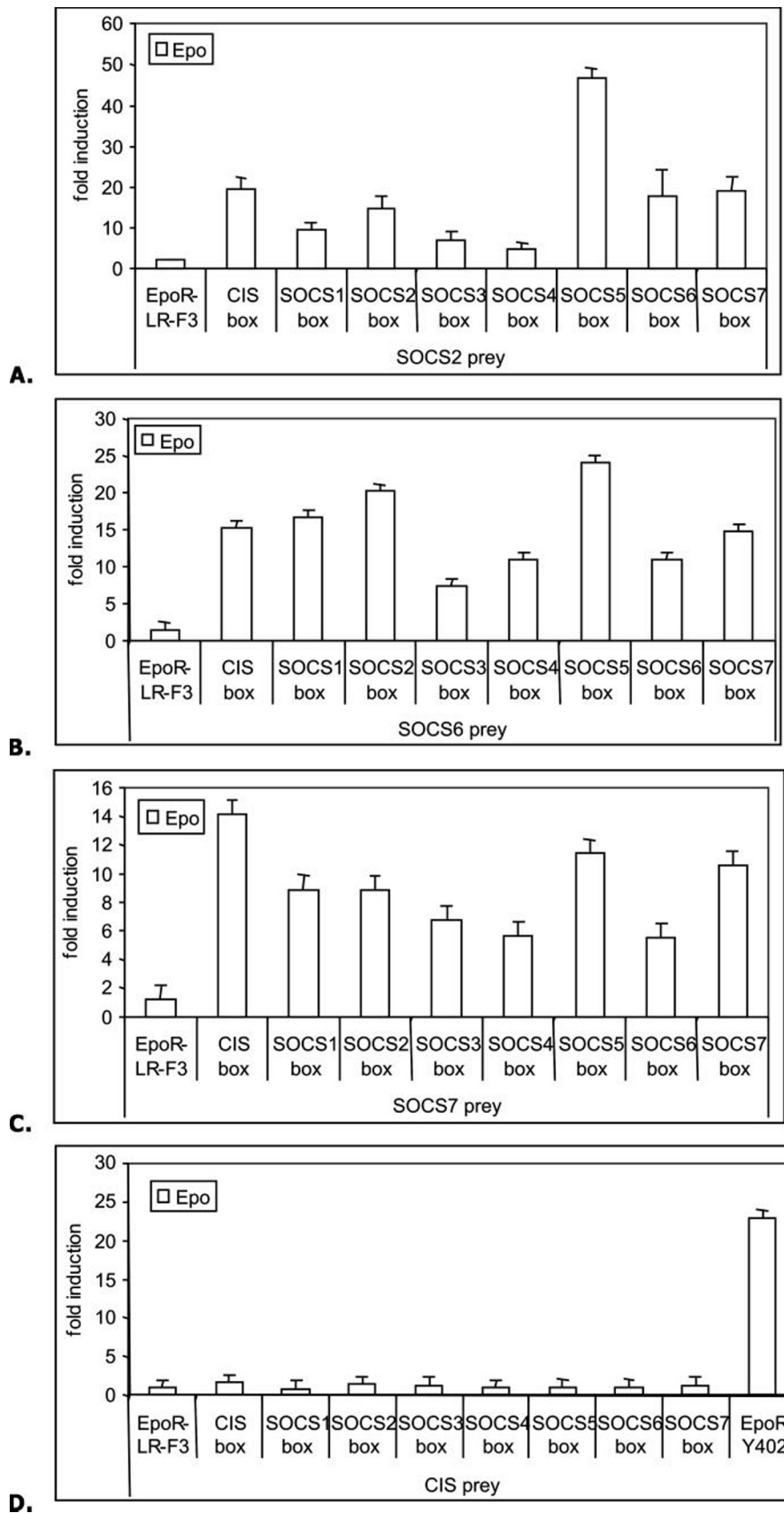


FIGURE 8. **SOCS2, SOCS6, and SOCS7 interact with the SOCS boxes of all SOCS family members.** HEK293-T cells were transiently cotransfected with plasmids encoding bait variants of the SOCS boxes of all SOCS proteins or the chimeric EpoR-LR-F3 construct as a negative control; the pMG2-SOCS2, pMG2-SOCS6, pMG2-SOCS7, or pMG2-CIS prey; and the pXP2d2-rPAP1-luciferase reporter. After transfection, cells were either left untreated or were stimulated with erythropoietin (Epo) for 24 h. Luciferase activities were measured in triplicate. Data are expressed as -fold induction (stimulated/non-stimulated).

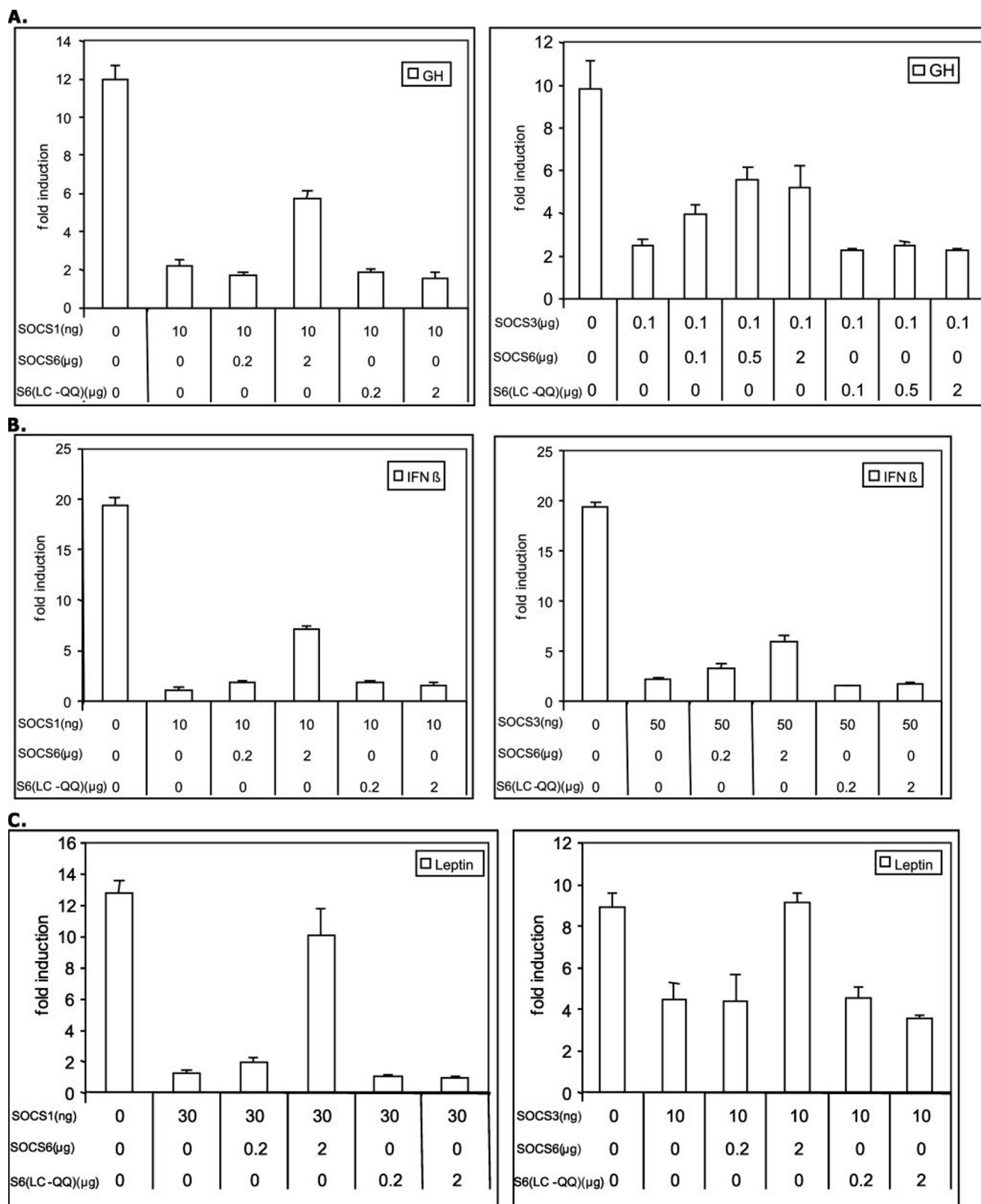


FIGURE 9. The negative effect of SOCS6 on other SOCS proteins depends on recruitment of the elongin BC complex. HEK293-T or N38 cells were transiently transfected with plasmids encoding SOCS1 or SOCS3 at fixed amounts and wild-type SOCS6 or SOCS6ΔBC box(LC-QQ) (S6Δ(LC-QQ)) at increasing concentrations. The transfected cells were either left untreated or were stimulated with GH (200 ng/ml), IFN-β (100 pM), or leptin (100 ng/ml). Data are expressed as fold induction (stimulated/non-stimulated). Luciferase and SEAP measurements were performed in triplicate. **A**, HEK293-T cells were transfected with 10 ng of SOCS1, 100 ng of SOCS3, and increasing concentrations of SOCS6 derivatives. GH signaling was assayed as described in the legend to Fig. 1. **B**, HEK293-T cells were transfected with 10 ng of SOCS1, 50 ng of SOCS3, and increasing concentrations of SOCS6 derivatives. IFN signaling was assayed as described in the legend to Fig. 2. **C**, N38 cells were transfected with 30 ng of SOCS1, 10 ng of SOCS3, and increasing concentrations of SOCS6 derivatives. Leptin signaling was assayed as described in the legend to Fig. 3.

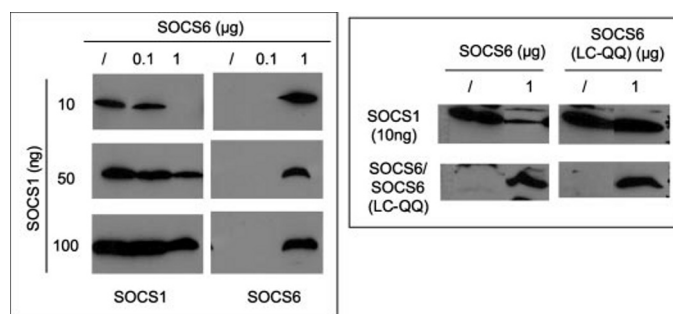


FIGURE 10. SOCS6, but not SOCS6(LC-QQ), promotes degradation of SOCS1. Increasing concentrations of FLAG-tagged SOCS6 or SOCS6(LC-QQ) were coexpressed transiently in HEK293-T cells with FLAG-tagged SOCS1. Cells were treated with cycloheximide (20 μ M) for up to 6 h. The lysates were blotted for SOCS1 and SOCS6 expression with anti-FLAG antibody.

stimulation and is more prolonged (27, 28, 32). Accumulation of increasing levels of SOCS2 late after induction is consistent with a role in eliminating excess levels of SOCS proteins after receptor activation and may be involved in restoring cellular responsiveness for subsequent stimulation. Interestingly, SOCS2, SOCS6, and SOCS7 can also bind to themselves, suggesting the possibility of self-elimination. A full and global insight into the precise inhibitory effects will thus require careful analysis of the interaction pattern at the cytokine receptor, at the targeted SOCS protein, and at the level of self-interaction, bearing in mind the effect of the differences in binding affinities and relative expression levels of all components.

Whereas SOCS1–3 and CIS have been studied extensively, so far little is known about the physiological role of the other four SOCS proteins, SOCS4–7. We therefore analyzed matrix-wise all possible inter-SOCS interactions. The interaction map showed two characteristics: first, SOCS2 appears to bind to all SOCS proteins, including itself; and second, SOCS6 and SOCS7 display exactly the same binding profile.

In line with a SOCS-counteracting role of SOCS6, we have shown that its expression potentiates signaling via the GHR, IFN type I receptor, and LR in a way quite similar to what we observed for SOCS2. Similar data sets were also obtained for leptin signaling in the physiologically relevant N38 hypothalamic cell line. These novel findings regarding SOCS6 provide an explanation for the significant enhancement of glucose metabolism observed in SOCS6 transgenic mice (31). More evidence for a positive role for SOCS6 in cytokine signaling also comes from studies in *Drosophila melanogaster*, where SOCS44A (which is similar to SOCS6) was shown to enhance the activity of the growth factor receptor/mitogen-activated protein kinase (MAPK) signaling cascade, in contrast to SOCS36E (which is similar to SOCS5) (56).

Because several SOCS molecules can mediate similar regulatory effects, functional redundancy is not unlikely. This may be particularly true for (but not limited to) SOCS6 and SOCS7, which show high homology and similarity in binding specificity (45). Compensatory effects between cross-modulatory SOCS molecules may perhaps have an effect on the phenotypes of SOCS2-, SOCS6-, and SOCS7-deficient mice (23, 45, 57), warranting analysis of double knock-out mice, which may uncover additional physiological activities of particular SOCS proteins. Of note, the SOCS box is not limited to the SOCS protein fam-

ily, but at present, 128 proteins harboring a SOCS box have been described in the mammalian genome (according to the Pfam Database).

In summary, our findings point to the existence of a subfamily of SOCS proteins consisting of SOCS2, SOCS6, and SOCS7, capable of controlling SOCS protein stability. This functional cross-modulation between SOCS proteins requires the SOCS box, probably both as an inter-SOCS-binding domain and as a functional recruitment motif for elongin BC-containing E3 enzymes. The observation that several SOCS proteins do not act solely as inhibitors of cytokine signaling should be taken in consideration in the evaluation of gene knock-out studies and may be of relevance for several human pathologies.

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Note Added in Proof—After submission of this manuscript, a paper was published by Ouyang *et al.* (58) demonstrating positive effects of SOCS2 upon ectopic expression in C2C12 myoblasts.

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