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Suppressor of Cytokine Signaling 7 Inhibits Prolactin, Growth Hormone, and Leptin Signaling by Interacting with STAT5 or STAT3 and Attenuating Their Nuclear Translocation*

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We report here the role of one of the less studied members of the family of suppressors of cytokine signaling (SOCS), namely SOCS-7, in cytokine signaling. We demonstrate that SOCS-7 inhibits prolactin (PRL), growth hormone (GH), or leptin (LEP) signaling mediated through STAT3 and STAT5 in a dose-dependent manner. SOCS-7 also attenuated STAT3 and STAT5 signaling induced by overexpression of JH1, the catalytic subdomain of JAK2. Since SOCS-7 interacted with phosphorylated STAT3 or STAT5, we assumed that SOCS-7 acts at the level of STAT proteins. Indeed, we showed that SOCS-7 inhibits PRL- and leptin-induced STAT5 and STAT3 phosphorylation and prevented the nuclear translocation of activated STAT3. Taken together, our results indicate that SOCS-7 is a physiological dysregulator of PRL, leptin, and probably also GH signaling and that its mode of action is a novel variation of SOCS protein inhibition of cytokine-inducible STAT-mediated signal transduction.

One of the main pathways for the termination of cytokine-activated JAK[1]/STAT signaling is mediated by a family of proteins discovered nearly a decade ago, the suppressors of cytokine signaling (SOCS) (1–4). These proteins' modulatory activity is not restricted to the JAK/STAT pathway; it has also been reported for activities mediated by receptor tyrosine kinases, such as c-Kit and insulin receptor substrate (IRS)-1 to -4 (5–7). The current paradigm attributes the main function of SOCS to the targeting of the affected proteins for ubiquitination and subsequent degradation by the proteasome, although additional mechanisms are also involved (4–6). The SOCS family consists of eight proteins, termed SOCS-1 through -7 and CIS (cytokine-inducible SH2 protein). Whereas CIS and SOCS-1 through -3 have been studied extensively, others, in particular SOCS-7, have been given much less attention. The latter protein was first discovered by Matuoka *et al.* (8), who cloned its partial cDNA sequence and called it NAP-4 due to its ability to interact with the adaptor proteins Nck, Grb2 (Ash), and Phospholipase C-γ-1. More recently, full sequences of mouse, rat, and human SOCS-7 have been reported (GenBank™ NM_138657 for mouse; XP_213443 for rat; and see Ref. 9 for human). The NAP-4 sequence lacks the 127 N-terminal amino acids of SOCS-7 but has an additional segment of 34 amino acids (exon 4). SOCS-7 is constitutively expressed in several tissues, most strongly in the testis and brain (8, 10, 11), and its expression in leukocytes is stimulated by prolactin (PRL), growth hormone (GH), and interleukin-6 (10). Recently, SOCS-7 (and SOCS-6) has been shown to interact with IRS-2 and IRS-4 (11). However, to date, no report concerning the possible biological role of SOCS-7 has been published aside from our recent work showing that SOCS-7 interacts with the cytoskeleton proteins actin and vinexin and is present in cell membranes (9). This latter finding prompted us to study whether SOCS-7 affects biological activities mediated through the membrane-embedded receptors of three cytokines: GH and PRL, which transduce their signal mainly through the JAK2/STAT5 pathway, and leptin, transducing through JAK2/STAT3.

EXPERIMENTAL PROCEDURES

Materials—Recombinant ovine PRL, ovine GH, and human leptin were prepared as described previously (14, 15). Rabbit anti-SOCS-7 immunoglobulins were produced as described by Martens *et al.* (9). Anti-SOCS-7 antibody, which also recognizes NAP-4, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and horseradish peroxidase-conjugated antibodies for Western blot analysis were purchased from Enco Co. (Jerusalem, Israel) and from Amersham Biosciences. SDS-PAGE reagents were from Bio-Rad, ECL reagents for Western blot analysis were from Amersham Biosciences or PerkinElmer Life Sciences, fetal calf serum was from Bet Haemek Co. (Jerusalem, Israel) or from Invitrogen (Merelbeke, Belgium), and luciferin was from Promega (Madison, WI). Plasmid encoding human leptin receptor (LEPR) was obtained from Dr. M. Rubinstein (Weizmann Institute), plasmids encoding human PRLR and STAT5-responsive luciferase (16) were from Drs. P. A. Kelly and V. Goffin (Faculty of Medicine Necker, Paris, France), and the STAT3-responsive pAH32 luciferase-encoding plasmid was from Merck. Restriction enzymes were purchased from Fermentas MBI (Vilnius, Lithuania),

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[1] The abbreviations used are: JAK, Janus kinase; SH2, Src homology 2; EMSA, electrophoretic mobility shift assay; GH, growth hormone; GST, glutathione *S*-transferase; HA, hemagglutinin; HEK, human epithelial kidney; hSIE, c-*cis*-inducible element of the human c-*fos* gene; IRS, insulin receptor substrate; JH1, catalytic subdomain of JAK2; LEP, leptin; LEPR, leptin receptor; LHRE, lactogenic hormone responsive element; NAP-4, shortened SOCS-7 or Nck-, Ash-, and phospholipase C-γ-binding protein 4; PIAS, protein inhibitor of activated STAT; PRL, prolactin; PRLR, prolactin receptor; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; YFP, yellow fluorescent protein; AD, activation domain; BD, binding domain.

Opti-MEM was from Invitrogen, and FuGENE 6 was from Roche Applied Science. Molecular weight markers for SDS-PAGE, Dulbecco's modified Eagle's medium, and Dulbecco's modified Eagle's medium/Ham F-12 medium were obtained from Invitrogen or Sigma, and SDS-PAGE reagents were from Bio-Rad. Other materials were from Sigma. Plasmids encoding human NAP-4 and hemagglutinin (HA)-tagged SOCS-7 were prepared as described previously (9). Plasmid encoding the catalytic subdomain of JAK2, GST-JH1, was received from Dr. A. Yoshimura (17), plasmid encoding pSVL-mSTAT3-YFP was from Dr. J. Tavernier (Ghent, Belgium), and plasmid encoding PEF-FLAG-I-mSOCS-7, originally cloned by D. Hilton, was a gift from Dr. P. De Sepulveda (Marseilles, France) (7).

In Vitro Bioassays in Transiently Transfected HEK-293T Cells—The effect of NAP-4- and SOCS-encoding constructs on PRL- and GH-inducible activity was assayed in HEK-293T cells transiently co-transfected with plasmids encoding ovine PRLR or ovine GH receptor, β-galactosidase, and the luciferase reporter gene, the latter controlled by a six-repeat sequence of the lactogenic hormone response element with a STAT5 binding sequence (LHRE) fused to a minimal thymidine kinase promoter (16, 18). The assay for leptin-inducible activity was carried out similarly using plasmids encoding the long form of hLEPRb and the STAT3-responsive pAH32 luciferase-encoding plasmid. JH1-dependent activity was determined similarly in cells transfected with 35 ng of GST-JH1-encoding plasmid. In all cases, the total amount of DNA was equalized by adding empty pcDNA vector. HEK-293T cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum as described previously (19), and experiments were carried out in six-well plates. HEK-293T cells were suspended to 125,000 cells/ml, plated at 2 ml/well and left at 37 °C (5% CO_2, 95% O_2) for 24 h. The transfection mixture, consisting of 1350 ng of the respective DNA, which always included 50 ng of β-galactosidase in Opti-MEM medium (100 μl), and Fugene 6 (6 μl), was preincubated at room temperature for 45 min and then gently added to each well. Cells were then incubated for an additional 24 h, and the respective hormones were added. Twenty-four hours later, the cells were lysed, and luciferase activity was determined and normalized for β-galactosidase activity and protein content as described previously (18, 19). To minimize transfection variability, each experiment was carried out in two or three plates, each containing six experimental treatments (see the legend to Fig. 1).

Yeast Two-hybrid Screening—Saccharomyces cerevisiae Y190 cells containing the two reporter genes *HIS3* and *lacZ* were transformed with pGBT9-NAP-4 and subsequently subjected to large scale transformations with 60 μg of a human leukocyte cDNA library fused to the Gal4 activation domain (AD) in the pACT2 vector (Clontech through BD Biosciences), according to the two-hybrid system TRAFO protocol (20). The expression of the Gal4-binding domain (BD) fusion protein was verified by Western blot using anti-Gal4-BD antibodies (Clontech), and identification of the interacting proteins was performed as described previously (9). All cDNA inserts encoding interacting proteins were identified by sequencing the whole insert using an Applied Biosystems (Foster City, CA) sequencer. Sequence analysis was performed by online BLAST searches.

Immunoprecipitation and Western Blotting—HEK-293T cells were transfected with plasmids encoding hPRLR or hLEPRb and with 0.5 μg of pCMV-HA-SOCS-7 plasmid DNA (1 μg in total) or empty vector as described above, and co-immunoprecipitation experiments were performed as described before (9).

Electrophoretic Mobility Shift Assay (EMSA)—HEK-293T cells were transfected with pCMV-HA-SOCS-7 plasmid DNA or empty pcDNA3.1 vector (1.5 μg in total) and leptin or PRL receptor (1 μg) as described above. Six hours post-transfection, cells were transferred to serum-free medium and incubated overnight. After stimulation with 100 ng/ml leptin or PRL for 30 min, cytoplasmic and nuclear proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's directions. Nuclear protein extracts (10 μg) were incubated with [α-^{32}P]dATP (3000 Ci/mmol; Amersham Biosciences) end-labeled double-stranded oligonucleotides (2–3 × 10^4 counts/min), corresponding to the c-*cis*-inducible element of the human c-*fos* gene (hSIE, m67 variant, sequences: 5′-GATCCGGGAGGGATTTACGGG-3′ and 5′-CAGCATTTCCCGTAAATCCCTCCCGG-3′) for 20 min at room temperature. For STAT5 EMSA, oligonucleotides corresponding to the GAS element of the β-casein promoter (5′-CAGATTTCTAGGAATTC-3′ and 5′-GGATTTGAATTCCTAGAAATC-3′) were used. Binding buffer contained 50% (w/v) glycerol, 10 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 2.5 ng/μl poly(dI-dC). To reach a total volume of 20 μl, 3.5 μl of loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 50% glycerol) was added. Competition experiments were performed using a 100-fold excess of unlabeled double-stranded oligonucleotides that was incubated for 1 h on ice with the nuclear extracts prior to the addition of the labeled probe. Supershift assays were performed with 1 μg of rabbit anti-STAT3 or anti-STAT5 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Normal rabbit serum was used as a control. Samples were electrophoresed on 5% nondenaturing polyacrylamide gels. Gels were dried, and bands were visualized by autoradiography.

Pull-down Experiments—In order to test possible interaction of SOCS-7 and JH1, HEK-293T cells were co-transfected with plasmids (1 μg each) encoding GST-JHI and HA-SOCS-7 or HA-SOCS-1. Twenty-four hours post-transfection, cell lysates were prepared and incubated with a 50% suspension of GSH-agarose beads for 4 h at 4 °C. Beads were washed three times, and the bound material was eluted with the sample buffer and resolved by 12% SDS-PAGE. The complexes were analyzed by Western blotting with rat anti-HA antibodies (clone 3F10; Roche Applied Science) and rabbit anti-SOCS-7 antibodies.

Immunocytochemistry—HEK-293T cells (8 × 10^4) were plated on 13-mm glass coverslips coated with poly-L-lysine (M_r > 80.000, 100 μg/ml; Sigma) in 0.5 ml of Dulbecco's modified Eagle's medium plus 10% (w/v) fetal calf serum per well of a 24-well plate. After overnight incubation, 100 ng of pSVL-STAT3-YFP plasmid DNA was co-transfected with plasmid encoding hLEPR and empty vector (pcDNA 3.1) or SOCS-7-expressing plasmid as described earlier. After 24 h, cells were transferred to serum-free medium and stimulated with 100 ng/ml leptin for different times. Cells were washed twice with PBS, fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature, and washed three times with PBS. Coverslips were mounted with Vectashield mounting medium (Labconsult, Genval, Belgium) and viewed using an Axiophot fluorescence microscope (Zeiss, Jena, Germany).

RESULTS

In the initial stage of the present work, two variants of SOCS-7, namely NAP-4 and SOCS-7, were tested for their ability to modulate STAT5-mediated ovine PRL- and ovine GH-inducible signaling as well as STAT3-mediated human leptin-inducible signaling. The respective hormone (ovine PRL, ovine GH, and human leptin) concentrations were chosen to achieve a submaximal response, as determined by preliminary experiments (not shown). The results (Fig. 1) clearly indicate that ovine PRLR-, ovine GH receptor-, and hLEPR-inducible luciferase activity is attenuated in a dose-dependent manner in HEK-293T cells transiently transfected with cDNA encoding either NAP-4 or SOCS-7. In the case of ovine PRL and GH, mediating STAT5 signaling, partial inhibition was already observed in cells transfected with 150 ng of NAP-4 or SOCS-7 (Fig. 1, *a* and *b*), and full inhibition was achieved by transfecting with 1000 ng of these constructs. It seems, therefore, that both ovine PRL- and ovine GH-inducible activity can be reduced by low concentrations of either NAP-4 or SOCS-7 (*i.e.* concentrations below the level of anti-NAP-4 or anti-SOCS-7 antibody detection as judged by Western blotting). The expression of the PRL, GH, and leptin receptors was not affected by co-expression of SOCS-7 (not shown). It should be noted that similar results were obtained using STAT-5-dependent alkaline phosphatase as the reporter gene (not shown). The sensitivity of the leptin R-mediated STAT3 signaling to NAP-4 and SOCS-7 was lower; even with 1000 ng of these constructs, only ~58 and 62% inhibition was detected, respectively (Fig. 1*c*). These differences could be partially attributed to the lower expression of NAP-4 and SOCS-7 in these experiments. To further explore the nature of the SOCS-7-dependent inhibition, we also tested whether receptor-independent autophosphorylation (due to overexpression) of the catalytic JH1 subdomain of JAK2 and subsequent activation of STAT5 and STAT3 signaling could be attenuated by SOCS-7. Strong inhibition was indeed observed (Fig. 2), indicating that once JAK2 is phosphorylated, the events affected by SOCS-7 do not require the presence of PRL, GH, or leptin receptors.

To test the possible interaction of SOCS-7 and JH1, we have performed a pull-down experiment using HEK 293T cells co-transfected with GST-JH1 and SOCS-7. The experiment was

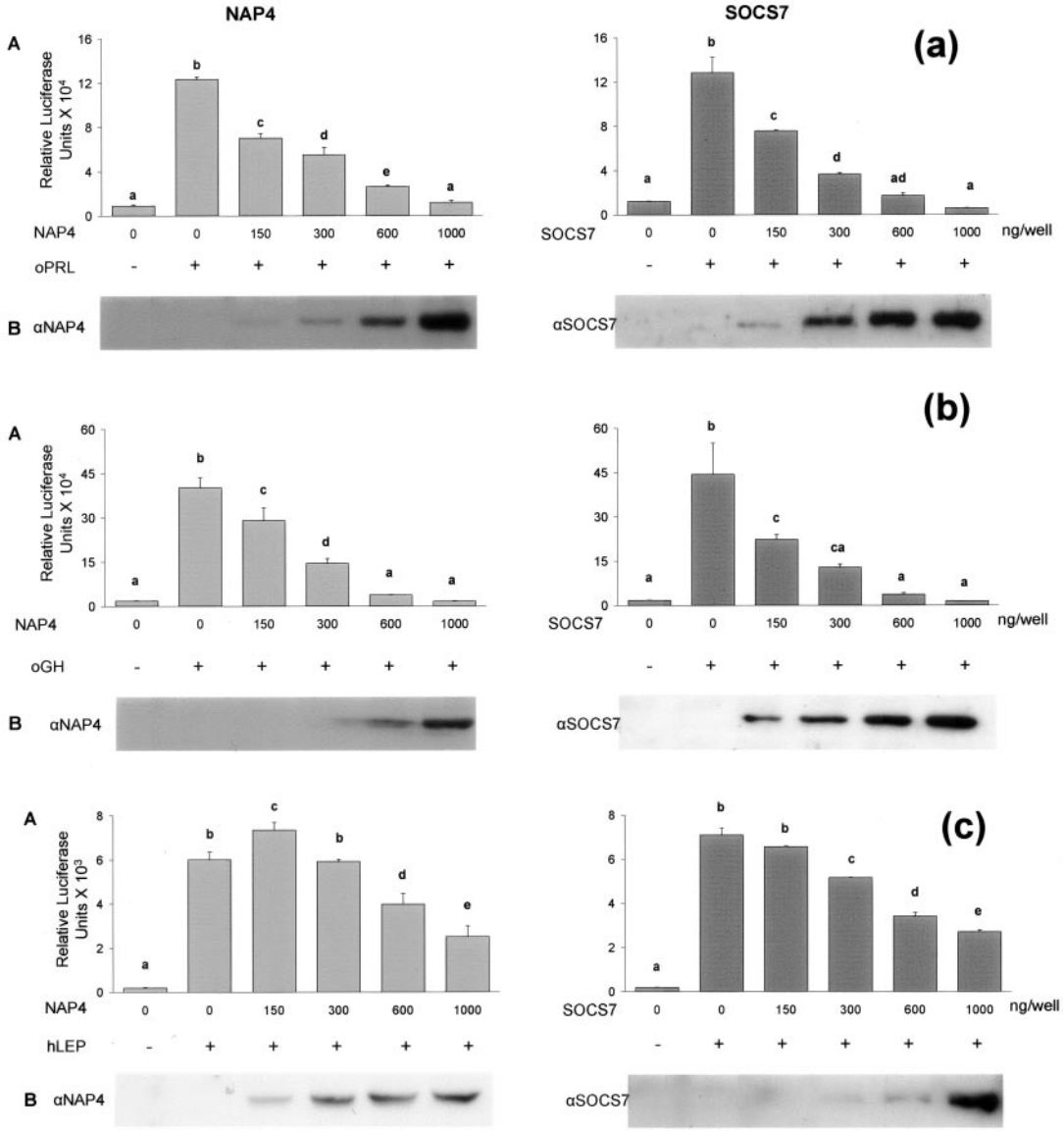

FIG. 1. **NAP-4 and SOCS-7 inhibit PRL and GH signaling through STAT5 and leptin-induced signaling through STAT3 in a dose-dependent manner.** HEK-293T cells were transfected with increasing amounts of plasmids encoding NAP-4 or SOCS-7 and 50 ng of β-galactosidase. Cells were also co-transfected with STAT5-responsive LHRE luciferase reporter plasmids (100 ng) along with plasmids (100 ng) encoding ovine PRL receptor (*a*) or ovine GH receptor (*b*) or with plasmids encoding STAT3-responsive pAH32 luciferase (250 ng) and leptin receptor (*c*) (250 ng). The total amount of DNA was equalized by adding empty pcDNA vector. Thirty-two hours post-transfection, cells were transferred to serum-free medium and treated with 100 ng/ml ovine (*o*) PRL (*a*), ovine GH (*b*), or human LEP (*c*) for 16 h. The graphs show a representative experiment (one of three), and the results are given as the ratio of luciferase to β-galactosidase activity expressed in relative luciferase units (*RLU*). The *bars* not marked with the same letter differ significantly ($p < 0.05$). Total cell lysates were immunoblotted with anti-NAP-4 or anti-SOCS-7 antibodies.

repeated three times. Although the SOCS-7 was identified in the total cell lysate, it was absent in the pull-down extract (not shown). The validity of the method was verified in a control experiment in which SOCS-7 was replaced by SOCS-1. In this experiment, SOCS-1 was found in both total cell lysate and pull-down extract as expected (not shown).

To explore the mechanism underlying the inhibition, we first utilized a yeast two-hybrid screen to search for protein partners of SOCS-7, using Gal4-BD-coupled NAP-4 and a Gal4-AD-coupled leukocyte cDNA library as bait (9). The interaction was assayed in *S. cerevisiae* Y190 yeast cells based on *HIS3* and *lacZ* reporter gene expression, the latter resulting in blue staining in a β-galactosidase production assay in the case of interaction (Fig. 3). One of the isolated clones encoded STAT3α missing 21 nucleotides at the 5′-end. Negative control interactions were tested between Gal4-BD (empty) and STAT3α-AD on the one hand and between Gal4-AD (empty) and NAP-4-BD on the other. The results, indicating an interaction between NAP-4 and STAT3, prompted us to continue this work at the protein level. However, we used only SOCS-7 for these experiments, because it is likely that SOCS-7, and not NAP-4, is the real physiological inhibitor of cytokine signaling. Indeed, in contrast to SOCS-7 (11), there are no indications of NAP-4 expression. As a first step, we tested whether leptin-inducible STAT3 and PRL-inducible STAT5 phosphorylation could be attenuated by co-transfection with SOCS-7, using FLAG-tagged SOCS-6 as a control. The results clearly showed SOCS-7 dose-dependent inhibition (Fig. 4, *a* and *c*), whereas no such inhibition could be seen in cells transfected with SOCS-6 (Fig. 4, *b* and *d*), the closest relative to SOCS-7. Since Akt (protein kinase B) is known to be phosphorylated subsequent to ligand stimulation of leptin R (21), we tested whether this stimulation

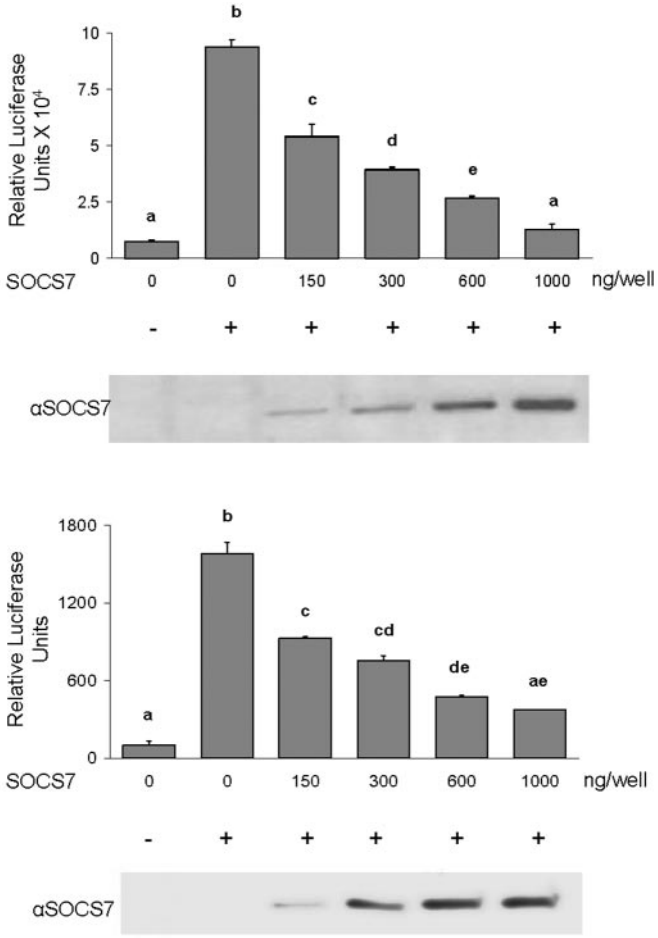

FIG. 2. **SOCS-7 inhibits JH1-signaling mediated through STAT5 and STAT3 in a dose-dependent manner.** HEK-293T cells were transfected with plasmids encoding various amounts of SOCS-7, 50 ng of β-galactosidase, 35 ng of GST-JH1 (marked as +), and 100 ng of STAT5-responsive LHRE luciferase reporter plasmid (*A*) or 250 ng of STAT3-responsive pAH32 luciferase reporter plasmids (*B*). Cells were treated as in the legend to Fig. 1 but without the hormones. The graphs show a representative experiment (one of two). For other details, see the legend to Fig. 1.

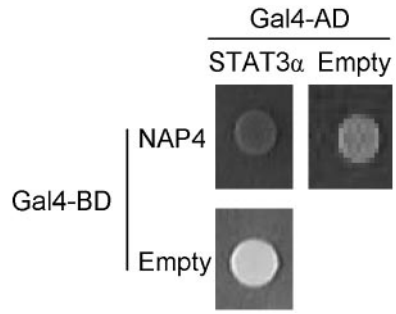

FIG. 3. **NAP-4 interacts with STAT3α in the yeast two-hybrid system.** Yeast two-hybrid interactions between Gal4-BD-coupled NAP-4 and Gal4-AD-coupled full-length STAT3α were assayed in *S. cerevisiae* Y190 yeast cells based on *HIS3* and *lacZ* reporter gene expression, the latter resulting in blue staining in a β-galactosidase production assay in the case of an interaction. As a control, interactions were tested between Gal4-BD (*Empty*) and STAT3α on one hand and between Gal4-AD (*Empty*) and NAP-4 on the other hand, and neither showed β-galactosidase staining.

could also be attenuated by SOCS-7 and found no inhibition at all (not shown). To further explore the nature of SOCS-7/STAT3 and SOCS-7/STAT5 interactions, co-immunoprecipitation experiments were carried out, showing that the formation of SOCS-7/STAT3 or SOCS-7/STAT5 complexes is clearly dependent on prior PRL- or leptin-induced STAT phosphorylation (Fig. 5, *a* and *b*).

As a consequence of complex formation between SOCS-7 and phosphorylated STAT3 or STAT5, the DNA binding of both STATs (extracted from the nuclei) was attenuated in a dose-dependent manner. This was documented by EMSA using cells transfected with both PRLR/STAT5 and LEPR/STAT3 and validated by respective supershift experiments (Fig. 6, *a* and *b*) performed on nuclear protein extracts. The specificity of the assay was further demonstrated by using cells transfected with SOCS-6·STAT3 and stimulated by leptin in a similar manner (Fig. 6*c*). Inhibition of nuclear transfer was also demonstrated by using STAT3 tagged with YFP (Fig. 7). HEK-293T cells transfected with STAT3-YFP and SOCS-7 (or empty vector) were stimulated with leptin (100 ng/ml) for up to 2 h. Prior to leptin stimulation, the fluorescence was diffusely distributed in the cytoplasm and nucleus and unaffected by SOCS-7 overexpression. Ten minutes after the addition of leptin, STAT3-YFP was mainly present in a patchy pattern in the nucleus of cells transfected with empty plasmid. In contrast, in cells overexpressing SOCS-7, STAT3-YFP was retained in the cytosplasm. After 30 min of leptin stimulation, the patchy distribution of STAT3 proteins in the nucleus of control cells increased, whereas in most SOCS-7-transfected cells, STAT3-YFP remained spread in the cytoplasm and was hardly seen in the nucleus. Two hours after leptin treatment, the patchy pattern was replaced by diffuse localization in the nucleus of most control cells as well as in SOCS-7-overexpressing cells. The specificity of SOCS-7 was further evidenced by using cells co-transfected with LEPR, STAT3, and either CIS or SOCS-6. In those cells, leptin-induced nuclear translocation of STAT3 was not affected by either CIS or SOCS-6 (not shown).

DISCUSSION

The results presented in this paper clearly demonstrate that co-expression of SOCS-7 with PRLR, GH receptor, or LEPR leads to inhibition of the respective ligand-inducible signaling mediated through STAT5 or STAT3. This is the first demonstration of the biological activity of SOCS-7, and it is clearly a dose-dependent phenomenon. Considering that its expression is induced by PRL and GH in leukocytes (9), our present results indicate that SOCS-7 is one of the physiological dysregulators of PRL and GH signaling, at least in those cells. It should be noted that the inhibition occurred already in cells transfected with low amounts of SOCS-7 (150 ng) in which the expression of the SOCS-7 protein was hardly visible, suggesting that SOCS-7 dependent attenuation of PRL-, GH-, and leptin-mediated activity may occur even at low, most likely physiologically relevant levels of SOCS-7 expression. To date, no information on leptin-inducible expression of SOCS-7 has been reported; therefore, the physiological significance of our results is still not clear with respect to this cytokine. However, since many cells express LEPR, PRLR, or GH receptor, PRL- or GH-inducible negative transregulation of leptin activity cannot be excluded. Furthermore, considering that SOCS-7 expression is highest in the testis (6) and that leptin, GH, and PRL play important roles in testis physiology (22, 23), our findings are indicative of a putative role for SOCS-7 in this organ.

Our finding that SOCS-7 also inhibits the activation of STAT5 and STAT3 transduction pathways activated by overexpression of the catalytic JH1 subdomain of JAK2 suggests that SOCS-7-inducible inhibition is mainly, if not entirely, a postreceptor phenomenon. Such inhibition of JH1-inducible STAT5 signaling has also been observed with SOCS-1 and SOCS-3 and, to a certain extent, CIS, but not with SOCS-2 or

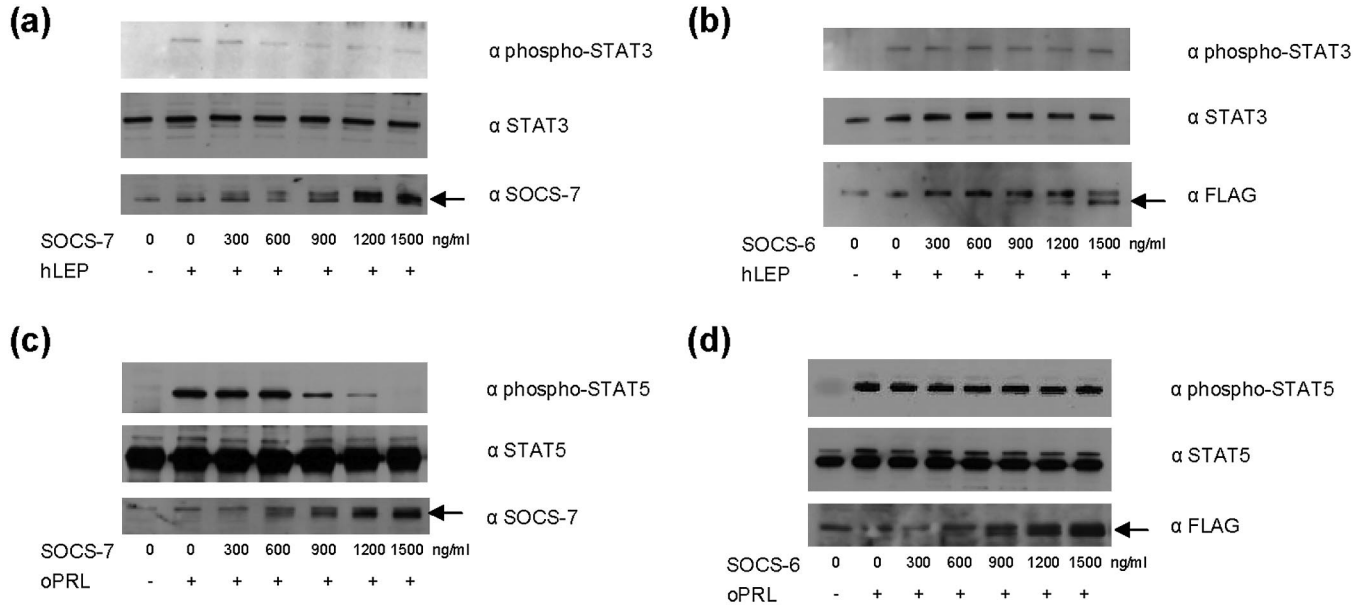

FIG. 4. **SOCS-7, but not SOCS-6, inhibits leptin- and PRL-induced tyrosine phosphorylation of STAT3 and STAT5, respectively, in a dose-dependent manner.** HEK-293T cells were co-transfected with plasmids encoding human leptin receptor (*a* and *b*) or ovine PRL receptor (*c* and *d*). Different amounts of SOCS-7 (*a* and *c*) or FLAG-SOCS-6 (*b* and *d*) cDNA were co-transfected together with empty vector to keep the total amount of cDNA (SOCS + empty) equal to 1.5 μg. Twenty-four hours post-transfection, cells were transferred to serum-free medium and stimulated with either 100 ng/ml human leptin (*hLEP*; *a* and *b*) or ovine PRL (*oPRL*; *c* and *d*) for 30 min as indicated. Cytoplasmic extracts were resolved by 10% SDS-PAGE and analyzed by Western blotting with anti-phosphotyrosine-STAT3 (*a* and *b*, *upper panel*) or STAT5 antibodies (*c* and *d*, *upper panel*). Blots were stripped and incubated with anti-STAT3 (*a* and *b*, *middle panel*) or STAT5 antibodies (*c* and *d*, *middle panel*). Western blotting was also performed with anti-SOCS-7 (*a* and *c*, *lower panel*) or anti-FLAG (*b* and *d*, *lower panel*) antibodies.

SOCS-4.[2] Interactions of SOCS-1 with JAK2 and its JH1 subdomain are well documented (7). To test whether this inhibition results from direct JH1 and SOCS-7 interaction, we have performed pull-down experiments using GST-JH1 and SOCS-7-HA and got negative results. Since the method was validated with SOCS-1-HA, we can conclude that the inhibition of JH1-inducible STAT5 signaling did not result from direct JH1-SOCS-7 interaction.

Our results clearly demonstrate that SOCS-7 prevents in part the phosphorylation of STAT5 and STAT3 (Fig. 4) but also interacts with the phosphorylated form of those proteins (Fig. 5). Those results appear paradoxical, unless SOCS-7 acts in two parallel or successive steps; 1) it blocks in part phosphorylation of STAT5 and STAT3 induced by JAK2 (which becomes phosphorylated due to PRL or leptin stimulation) or by phosphorylated JH1 (which becomes phosphorylated due to overexpression) in a not yet understood manner, and 2) both phosphorylated STATs interact with SOCS-7, and this interaction prevents their translocation to the nucleus. The latter step was documented here by EMSA and respective supershift experiments (Fig. 6) as well as by a determination of the subcellular localization of YFP-STAT3 proteins using fluorescence microscopy (Fig. 7). The interaction between SOCS-7 and STAT3 has also been observed in yeast (see Fig. 3), although STATs most probably cannot be phosphorylated in that system. Therefore, this interaction could be forced by the overexpression of both fusion proteins.

The STAT3/STAT5 interaction with SOCS-7 is unique and indicative of a novel variation in SOCS signaling, which has not been reported for other SOCS proteins, namely that SOCS-7 acts as both conventional SOCS and protein inhibitor of activated STAT (PIAS). However, unlike PIAS proteins, which inhibit STATs by either preventing their interaction with DNA or activating translation (25), SOCS-7 acts in the cytoplasm. Furthermore, in preliminary experiments, we have observed that in

[2] A. Gertler and Y. Cohen, unpublished data.

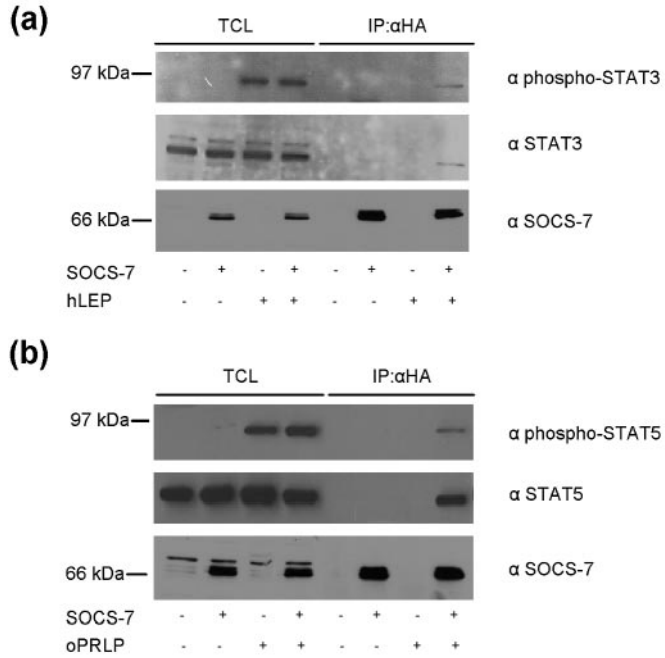

FIG. 5. **SOCS-7 interacts with activated STAT3 and STAT5 in mammalian cells as shown by co-immunoprecipitation.** HEK-293T cells were transfected with 0.5 μg/ml HA-SOCS-7 (+) or empty vector (−) and plasmids encoding human leptin receptor (*a*) or ovine PRL receptor (*b*). Twenty-four hours post-transfection, cells were stimulated with 100 ng/ml hLEP (*a*) or oPRL (*b*) for 30 min as indicated. Total cell lysates (*TCL*) or immunoprecipitates with anti-HA antibodies (*IP*:*αHA*) were subjected to Western blot analysis. Endogenously expressed phospho-STAT3 (*a*) or phospho-STAT5 (*b*) proteins were co-precipitated with SOCS-7 as shown by Western blotting with anti-phospho-STAT3 (*a*, *upper panel*) or anti-phospho-STAT5 (*b*, *upper panel*) antibodies. The blot was stripped and reprobed with anti-STAT3 (*a*, *middle panel*) or anti-STAT5 (*b*, *middle panel*) antibodies. Western blotting with rabbit anti-SOCS-7 γ-globulins was performed to show expression and precipitation of overexpressed SOCS-7 (*a* and *b*, *lower panel*).

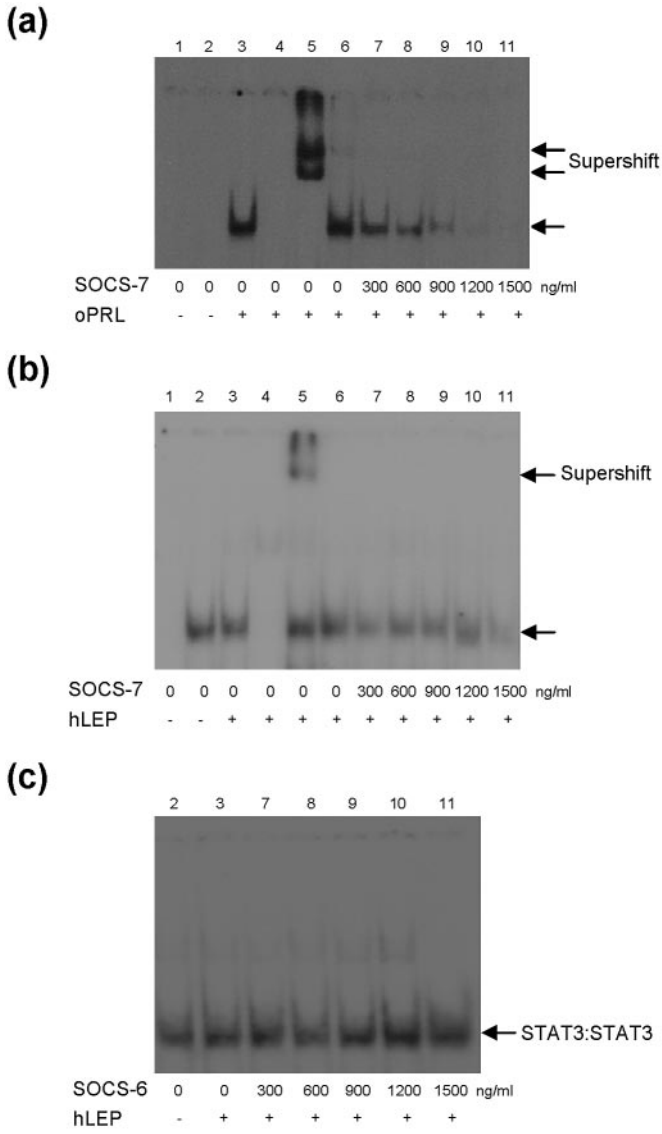

FIG. 6. **Dose-dependent inhibition of STAT3 and STAT5 nuclear DNA binding activity by SOCS-7.** HEK-293T cells were co-transfected with plasmids coding for PRL receptor (*a*) or leptin receptor (*b* and *c*). Different amounts of SOCS-7 (*a* and *b*) or SOCS-6 (*c*) cDNA were co-transfected together with empty vector to keep the total amount of cDNA (*SOCS + empty*) equal to 1.5 μg. Twenty-four hours post-transfection, cells were stimulated with 100 ng/ml ovine PRL (*oPRL*; *a*, *lanes 3–11*) or human leptin (*hLEP*; *b*, *lanes 3–11*; *c, lanes 3* and *7–11*) for 30 min. Nuclear extracts were subjected to EMSA with a probe corresponding to the GAS element of the β-casein promoter (*a*) and the hSIE m67 probe (*b* and *c*) and resolved on a 5% acrylamide gel. Labeled probe alone was run in the *first lane* and migrated out of the gel (*a* and *b*, *lane 1*). Competition experiments were performed with a 100-fold excess of free probe (*a* and *b*, *lane 4*). Supershift DNA-protein complexes were obtained with anti-STAT5 (*a*, *lane 5*) or anti-STAT3 (*b*, *lane 5*) antibodies. Normal rabbit serum was used as control for the supershifts (*a* and *b*, *lane 6*).

Jurkat T-lymphoma cells stimulated with hIFNα, phosphorylated STAT3 (in contrast to nonphosphorylated STAT3) was immunoprecipitated by anti-SOCS-7 antibodies, whereas no such immunoprecipitation was observed in the absence of hIFNα,[3] indicating that SOCS-7 and phosphorylated STAT3 interaction is a physiological phenomenon.

The SOCS-7/STAT3 interaction leading to attenuation of the latter's nuclear signaling is quite specific, since leptin-induced phosphatidylinositol 3-kinase-mediated Akt phosphorylation,

[3] N. Martens and E. L. Hooghe-Peters, unpublished data.

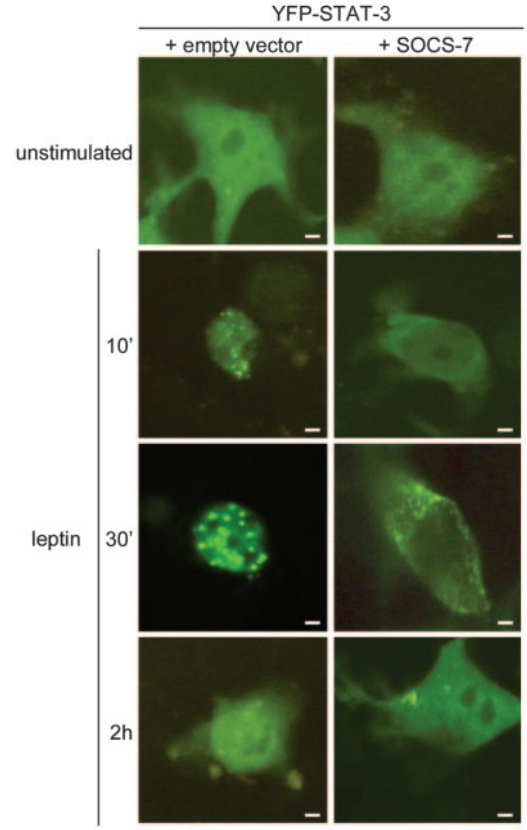

FIG. 7. **SOCS-7 inhibits leptin-induced nuclear translocation of STAT3.** HEK-293T cells were grown on coverslips and co-transfected with plasmids encoding human leptin receptor, YFP-STAT3, along with plasmid encoding SOCS-7 or empty vector. Twenty-four hours post-transfection, cells were transferred to serum-free medium and stimulated with human leptin (100 ng/ml) for 30 min, fixed with 4% paraformaldehyde, and analyzed for subcellular localization of YFP-STAT3 proteins using a fluorescence microscope. *Scale bars*, 5 μm.

which is not dependent on STAT3 (21), is not affected by SOCS-7. Since Akt phosphorylation is the final reaction in a chain initiated by SH2-B, a JAK2-interacting protein that promotes activation of the phosphatidylinositol 3-kinase pathway by recruiting IRS-1 and IRS-2 in response to leptin (21), we also speculate that the putative interaction of JAK2 with SOCS-7 does not preclude its interaction with SH2-B. Thus, the fact that the IRS-2 pathway was not affected suggests that either the reported interaction between SOCS-7 and IRS-2 (6) is not biologically relevant, at least in this system, or redundant pathways are available. Our data also do not support suggestions, based on sequence homology and present biochemical observations, that SOCS-6 and SOCS-7 have similar and potentially redundant functions (6).

Although the nature of the interaction between SOCS-7 and STAT3 or STAT5 is not known, it should be noted that the recognition sites of phosphopeptides for interaction with STAT1 or STAT3 are, respectively, pY(D/E)(P/R)(Q/P/R) and pY(basic/hydrophobic)(P/basic)(Q) (26) and that the consensus motif for interaction with STAT5 is pYL*X*(I/L/V) (27). Interestingly, both human and mouse SOCS-7, in contrast to other SOCSs, contain the YDPQ sequence (aa 554–557), a YIRK sequence (aa 547–550), which, when tyrosine-phosphorylated, is similar to the STAT3 binding motif, and the YLSL sequence (aa 561–564), which is identical to the STAT5 binding motif. Although phosphorylation of SOCS-7 has not been documented, tyrosine phosphorylation of other SOCS factors has been described (28). We have also observed SOCS-7 and unphosphorylated STAT5 interaction in a yeast two-hybrid exper-

iments, but this could be due to overexpression.

Recently, we reported on an interaction between the cytoskeletal component vinexin and SOCS-7 (9). Since the inhibitory activity of several SOCS factors (SOCS-1, 2, and 3) on GH-induced STAT5 reporter activity is dependent on an intact actin cytoskeleton (29), we suggest that this also may apply to SOCS-7, making it the putative agent linking STAT3 and STAT5 to the cytoskeleton. In conclusion, SOCS-7 is a *bona fide* suppressor of cytokine signaling. However, it also acts as a protein inhibitor of activated STAT5 and STAT3 and abolishes their translocation to the nucleus. The physiological role of SOCS-7 was most recently confirmed by reporting generation of SOCS-7 knock-out mice (30). The KO mice were 10% smaller than their wild-type littermates, and a large proportion of them developed hydrocephalus. *In situ* hybridization studies confirmed prominent expression of SOCS-7 in the brain, suggesting a role for SOCS-7 in this organ (30).

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