Biological Evidence That SOCS-2 Can Act Either as an Enhancer or Suppressor of Growth Hormone Signaling*

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Suppressor of cytokine signaling (SOCS)-2 is a member of a family of intracellular proteins implicated in the negative regulation of cytokine signaling. The generation of SOCS-2-deficient mice, which grow to one and a half times the size of their wild-type littermates, suggests that SOCS-2 may attenuate growth hormone (GH) signaling. In vitro studies indicate that, while SOCS-2 can inhibit GH action at low concentrations, at higher concentrations it may potentiate signaling. To determine whether a similar enhancement of signaling is observed in vivo or alternatively whether increased SOCS-2 levels repress growth in vivo, we generated and analyzed transgenic mice that overexpress SOCS-2 from a human ubiquitin C promoter. These mice are not growth-deficient and are, in fact, significantly larger than wild-type mice. The overexpressed SOCS-2 was found to bind to endogenous GH receptors in a number of mouse organs, while phosphopeptide binding studies with recombinant SOCS-2 defined phosphorylated tyrosine 595 on the GH receptor as the site of interaction. Together, the data implicate SOCS-2 as having dual effects on GH signaling in vivo.

The suppressor of cytokine signaling (SOCS)1 proteins are a family of eight SH2 domain-containing proteins, comprising cytokine-inducible SH2 domain-containing protein (CIS) and SOCS-1-7. Studies in many laboratories have implicated SOCS proteins in the attenuation of cytokine action through inhibition of the Janus kinase (JAK)/signal transducer and activators of transcription (STAT) signal transduction pathway. SOCS proteins operate as part of a classical negative feedback loop, in which activation of cytokine signaling leads to their expression. Once produced, SOCS proteins bind to key components of the signaling apparatus to prevent further signal transduction and possibly target them for degradation via a conserved C-terminal motif, called the SOCS Box, that recruits ubiquitin ligases (reviewed in Refs. 1–3).

While in vitro studies have suggested that SOCS proteins may be promiscuous in their activity, gene deletion studies in mice have highlighted their importance in a limited number of signaling pathways. SOCS-1 is a key regulator of interferon γ signaling, T-cell homeostasis, and lactation (4–6), while SOCS-3 is thought to play a crucial role in placentation function (7). CIS-deficient mice are reported to have no phenotype, although CIS transgenic mice display growth retardation and defects in mammary development which are accompanied by reductions in STAT5 phosphorylation (8). Interestingly, this phenotype has similarities to those observed in STAT5a- and STAT5b-deficient mice (9–11).

SOCS-2-deficient animals exhibit accelerated post-natal growth resulting in a 30–50% increase in body weight by 12 weeks of age, significant increases in bone and body lengths, thickening of the skin due to collagen deposition, and increases in internal organ size (12). This phenotype has striking similarities to those of insulin-like growth factor (IGF)-I and growth hormone (GH) transgenic mice (13, 14), as well as the high growth mutant mouse (15). Further investigation of the SOCS-2−/− phenotype identified significant increases of IGF-1 mRNA in some tissues and lower levels of major urinary protein, the expression of which is regulated by pulsatile GH secretion (12). Recently, STAT5 phosphorylation in response to GH has been shown to be modestly prolonged in SOCS-2−/− primary hepatocytes compared with those from wild type mice, and much of the acceleration of growth in SOCS-2−/− mice requires the presence of STAT5b, a key mediator of GH action (16).

The biochemical mechanism by which SOCS-2 suppresses GH action remains largely unknown. SOCS-2 mRNA expression is induced by GH (17, 18), but overexpression studies in vitro to define the effects of SOCS-2 on GH action have been ambiguous. Some reports show SOCS-2 to moderately inhibit GH signaling (19, 20), others suggest SOCS-2 may enhance signaling (18, 21), while others have shown SOCS-2 to suppress signaling at low doses and enhance signaling at higher doses (22). Here we report on the generation of mice that transgenically overexpress SOCS-2 and find that these mice display no apparent growth retardation as might be predicted, but rather are larger than wild-type littermates in a number of growth parameters. With these mice we demonstrate that SOCS-2 interacts with endogenous GH receptors in primary cells, and using synthesized peptides we have mapped an important site of SOCS-2 interaction to Tyr595 of the GH receptor.

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1 The abbreviations used are: SOCS, suppressor of cytokine signaling; CIS, cytokine-inducible SH2 domain-containing protein; JAK, Janus kinase; STAT, signal transducer and activators of transcription; IGF, insulin-like growth factor; GH, growth hormone; DME, Dulbecco’s modified Eagle’s medium; MT-PBS, mouse-tonicity phosphate-buffered saline; rh, recombinant human.
**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice**—SOC2-2 transgenic mice were generated by ligating the mouse SOCS-2 coding region into the pUbiFLAG vector (22), in which expression of the gene of interest is driven by the human ubiquitin C promoter (23). Transgenic mice were generated as previously described in Ref. 23. Mice carrying the SOCS-2 transgene were bred to establish whether they transmitted them to progeny, then Northern blotting was performed as described previously (24) on RNA from a range of tissues to evaluate expression.

**Growth Analysis**—Growth curves, organ weights, and bone measurements were performed as described previously (12).

**Analysis of Protein Expression/interaction in SOCS-2 Transgenic Mice**—Protein extracts and immunoprecipitation experiments from tissues were prepared and performed essentially as described (23), except that tissues were lysed in muscle lysis buffer (0.5% (v/v) Nonidet P-40, 50 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonfyl fluoride, 1 mM sodium fluoride, and protease inhibitors (Roche Molecular Biochemicals)), and 40 μg of total protein from each organ lysate was blotted on Western blots and probed to determine HSP70 levels using the antibody sc-24 (Santa Cruz). Antibodies against mouse GH receptor were kindly provided by P. Talamantes, University of California.

**Transient Transfections**—Transfections were performed in 293T cells plated at 2 × 10⁵ cells/ml in 2 ml of DME with 10% fetal calf serum using FuGENE transfection reagents (Roche Molecular Biochemicals). Briefly, 100 ng each of GH receptor plasmid, LHRE-luc reporter plasmid (kindly provided by Dr. Jane Visvader, Walter and Eliza Hall Institute of Medical Research), β-galactosidase plasmid, and 0–100 ng of pEF-SOCS-2 plasmid (23) made up to 250 ng with pEFBOS plasmid were transfected into a six-well plate 24 h after cells were plated out. Twenty-four hours later, cells were washed once with mouse toxicity phosphate-buffered saline (MT-PBS), and the culture medium was replaced with DME containing 1% bovine serum albumin. Cells were left to equilibrate for 1 h before 500 ng/ml of recombinant human (rh)GH was added to appropriate groups. Cells were incubated for a further 16 h before being lysed and assayed for luciferase and β-galactosidase activity as described (25).

**Histopathological Examination**—The heart, lungs, brain, skin, muscle, spleen, thymus, mesenteric lymph node, liver, kidneys, bladder, seminal vesicles, uterus, and testicles were collected from 12-week-old animals; weighed; and then fixed in 10% saline-buffered formalin before being sectioned, stained with hematoxylin/eosin, and analyzed as described previously (12).

**Generation of Recombinant SOCS-2 Proteins**—DNA encoding the murine SOCS-2 SH2 domain (amino acids 37–159) was amplified from the pEF-SOCS-2 plasmid by PCR and ligated into the pET-43.1 NusA fusion protein expression plasmid (Novagen). DNA encoding the hexa-His sequence was engineered into the 3′ primer to aid in purification. The vector was transformed into BL21(DE3) cells, and cultures were grown at 30°C until OD₆₀₀ was 0.6 units before being induced with 0.1 mM isopropyl-β-thiogalactopyranoside. Cells were harvested 2 h post-induction, and cell pellets were lysed in 10 ml of lysis buffer per 50 ml of culture (MT-PBS containing 0.2 mg/ml lysozyme, 1% Triton X-100, 1 mM phenylmethylsulfonfyl fluoride, and 30 μg/ml DNase I) for 60 min at 4°C. The total cell lysate was centrifuged for 10 min at 27,000 × g at 4°C. The supernatant was then loaded onto a nickel-nitrilotriacetic acid column (Qiagen) equilibrated in buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0), washed with buffer containing 10 mM β-mercaptoethanol and 10 mM imidazole eluted with 200 mM imidazole. Fractions were collected and then EDTA and β-mercaptoethanol were added to achieve final concentrations of 2 and 40 mM. Fractions that contained His-tagged proteins were pooled and applied to a size-exclusion chromatography column (Sephadex 200). Samples were subsequently analyzed by SDS-PAGE and Western blotting using anti-His antibodies (penta-His, Qiagen).

**Peptide Synthesis**—Synthetic peptides with a C-terminal amide were synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) amino acids activated with O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate. Peptides were biotinylated by coupling d-biotin to the N terminus of resin-bound peptides before cleavage and deprotection. Peptide products were purified by reverse phase-high performance liquid chromatography and analyzed by matrix-assisted laser desorption/ionization mass spectrometry.

**Recombinant SOCS-2 Protein/GH Receptor Peptide Interaction**—Biotinylated phosphopeptides were immobilized on streptavidin-agarose resin (Pierce, Brisbane, Australia) using standard procedures, and 20 μl of resin was incubated with 25 μg of NusA-SOCS-2-SH2 in 1 ml of Tris, pH 7.5, 0.1% Tween 20 for 2 h at 4°C. Beads were then washed twice with cold PBS, 0.1% (v/v) Tween 20 and boiled in 25 μl of 2× reducing loading buffer (125 mM Tris-HCL, pH 6.8, 20% (v/v) glycerol, 4% SDS, and 5% (v/v) β-mercaptoethanol). SDS-PAGE was performed on samples as described in Ref. 16.

**RESULTS**

**SOCS-2 Can Enhance and Suppress GH Signaling**—The effects of SOCS expression on GH-induced transcription were analyzed by transient transfections of 293T cells with a GH-responsive STAT5-dependent luciferase reporter, porcine GH receptor, and a β-galactosidase-containing plasmid in the presence or absence of plasmids encoding CIS, SOCS-1, SOCS-2, or SOCS-3. In the absence of exogenous SOCS proteins, GH stimulation resulted in a 16-fold increase of reporter activity. The expression of increasing levels of transfected SOCS-2 initially caused repression of reporter activity to 40% of that seen in the absence of SOCS, but at higher concentrations of SOCS-2 a recovery from inhibition and a significant enhancement of reporter activity were observed (Fig. 1). Expression of SOCS-1 and SOCS-3 prevented any significant reporter activity, and CIS was also a potent inhibitor of GH-induced activity.

**Transient Expression of SOCS-2 Does Not Inhibit Growth, but Enhances It**—To determine whether high levels of SOCS-2 could also potentiate GH signaling in vivo, we generated SOCS-2 transgenic mice. Three independent lines of mice that transmitted the SOCS-2 transgene were produced and two of these (line F33 and line F9) were analyzed further. Expression of SOCS-2 from the transgene in these lines was confirmed by immunoprecipitation of SOCS-2 protein via the FLAG epitope from lysates of all organs examined (Fig. 2A).

Given the accelerated growth in mice deficient for SOCS-2, it was interesting to observe that SOCS-2 transgenic mice suffered from no apparent growth retardation throughout the post-natal period but, rather, displayed enhanced growth. Male mice were significantly heavier from 3 weeks of age resulting in a 13–15% increase in body weight (Fig. 2B). This trend was confirmed in male organ weights with both lines, demonstrating an overall increase in the size of most organs and tissues, with some being significant enlarged, particularly the carcass (Fig. 2C). Significant changes were also found in the growth rates and body and organ weights of female transgenic mice, although they were of a lesser magnitude (data not shown).

**Histological and Hematopoietic Analysis**—Histological ex-
amination of 3-month-old mice from both transgenic lines compared with wild-type mice revealed no consistent abnormalities or defects in any organ or tissue examined. No differences were detectable in white blood cells, hematoctrit, or platelet numbers between groups, and normal numbers of progenitor cell-derived colonies were generated in cultures of marrow cells from transgenic mice when stimulated with a range of cytokines (data not shown). Despite data suggesting that the onset of blast crisis in patients with chronic myeloid leukemia is accompanied by the development of high levels of SOCS-2 mRNA (26), we found no indication that elevated SOCS-2 levels led either to leukemia development or to maturation arrest in hematopoietic cells of any lineage.

**SOCS-2 Interacts with the GH Receptor in Vivo**—Based on the hypothesis that SOCS-2 may regulate or bind to the GH receptor, immunoprecipitations of the SOCS-2 protein were performed from a number of tissues from wild-type and transgenic mice, before and after GH injection. These lysates were then electrophoresed and examined by Western blotting. As expected FLAG-tagged SOCS-2 was detected in transgenic but not wild-type mice, and this was observed to interact with the growth hormone receptor, especially after growth hormone injection (Fig. 3). A similar experiment was also performed where an animal was injected with IGF-I, and the Western blot was probed with antibodies against the IGF-I receptor, but no interaction was detected (data not shown).

**SOCS-2 Interacts with Tyrosine 595 of the Human GH Receptor**—Previous studies have shown that SOCS-2 can interact with the phosphorylated GH receptor in vitro (19, 21), and we have shown here an in vivo association. To further define the nature of this interaction, we designed biotinylated phosphorylated Tyr(P) peptides to each of the seven tyrosine residues phosphorylated on the human GH receptor in response to GH (Fig. 4A). These peptides were bound to streptavidin-Sepharose before being incubated with a recombinant fusion protein of NusA and the SH2 domain of SOCS-2. Significant phosphopeptide/fusion protein interaction was detected with the Tyr(P)595 peptide (Fig. 4B), and the specificity of the interaction was confirmed by incubating NusASOCS-2 SH2 protein with either phosphorylated or non-phosphorylated Tyr(P)595 peptide, and NusA recombinant protein with Tyr(P)595 peptide.

**DISCUSSION**

The observation that mice transgenically overexpressing SOCS-2 did not have repressed growth, but rather slightly enhanced growth, is somewhat surprising given the striking gigantism phenotype of SOCS-2−/− mice (12) and implies that SOCS-2 can also have a positive role in GH signaling. The evidence for SOCS-2 playing a positive role was first reported by Adams et al. (18) and later shown by the more detailed experiments by Favre and colleagues (22). This latter study demonstrated that low concentrations of SOCS-2 inhibited growth hormone action, while higher concentrations SOCS-2 enhanced signaling. A similar phenomenon has been observed with SOCS-2 expression and prolactin signaling, but has not been observed for SOCS-1 or SOCS-3 in any cytokine system (27). This observation has in vivo correlates, since at normal physiological levels SOCS-2 is presumed to have a net inhibitory effect, manifest by an increase in signaling in SOCS-2.
deficient mice, while the superphysiological levels of SOCS-2 obtained in the present transgenic mice implicate an accentuation of growth hormone signaling.

Several theories might be put forward to explain the dual effect of SOCS-2. First, overexpressed protein might not be completely functional and may act as a dominant negative. This is unlikely given that FLAG-tagged SOCS-2 can inhibit signaling in vitro and can interact with endogenous GH receptors in vivo. An alternative explanation would propose that SOCS-2 binds to different tyrosine-phosphorylated residues with different affinities. Results in this paper suggest that the high affinity binding site within the growth hormone receptor is Tyr595. Tyr595 has been implicated in negative regulation of signaling since receptors in which this residue is mutated to Phe exhibit enhanced signaling (28). In addition to SOCS-2, the cytoplasmic tyrosine phosphatase, SHP2, has also been shown to interact with Tyr595, and this interaction has been assumed to be responsible for attenuating signaling (28). Our data suggest that SOCS-2 may at least in part be responsible for negative regulation of growth hormone signaling through Tyr595, and further studies are required to assess the relative importance of SOCS-2 and SHP-2 in regulation of growth hormone signaling.

Interestingly, in vitro studies showed SOCS-3 to be a more profound inhibitor of GH-induced signaling than SOCS-2. SOCS-3 also binds to the cytoplasmic tail of the growth hormone receptor, although its preferred binding site is either Tyr487 (21) or Tyr332 (19). It will be interesting to determine whether SOCS-2 can bind with low affinity to either of these phosphorylated tyrosine residues. If this is the case, then the capacity of SOCS-2 to potentiate signaling may be due to its ability, at high concentrations, to compete with endogenous SOCS-3 for binding to these Tyr sites on the GH receptor. This model is consistent with the in vitro observation that increasing concentrations of SOCS-2 can overcome the effect of exogenous SOCS-1 and SOCS-3 on growth hormone activity and prolactin signaling (22, 29). Further biochemical analysis of the interaction between individual SOCS proteins and the GH receptor will aid in understanding the physiological mechanisms of growth regulation.

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FIG. 4. SOCS-2 interacts with Tyr595 of the GH receptor. A, diagram of the location of tyrosine residues phosphorylated in response to GH and the sequences of the synthesized phosphopeptides. B, immobilized phosphopeptides were incubated with recombinant SOCS-2 SH2 domain protein fused to the NusA protein, washed, separated on SDS-PAGE, and Coomassie-stained. The specificity of the SOCS-2 SH2 domain interaction was tested with non-phosphorylated Tyr595 and NusA with phosphorylated Tyr595.