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SOCS/CIS Protein Inhibition of Growth Hormone-stimulated STAT5 Signaling by Multiple Mechanisms*

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The inhibition of growth hormone (GH) signaling by five members of the GH-inducible suppressor of cytokine signaling (SOCS/CIS) family was investigated in transfected COS cells. Complete inhibition of GH activation of the signal transducer STAT5b and STAT5b-dependent transcriptional activity was observed upon expression of SOCS-1 or SOCS-3, while partial inhibition (CIS, SOCS-2) or no inhibition (SOCS-6) was seen with other SOCS/CIS family members. SOCS-1, SOCS-2, SOCS-3, and CIS each strongly inhibited the GH receptor (GHR)-dependent tyrosine phosphorylation of JAK2 seen at low levels of transfected JAK2; however, only SOCS-1 strongly inhibited the GHR-independent tyrosine phosphorylation of JAK2 seen at higher JAK2 levels. To probe for interactions with GHR, *in vitro* binding assays were carried out using glutathione *S*-transferase-GHR fusion proteins containing variable lengths of GHR's COOH-terminal cytoplasmic domain. CIS and SOCS-2 bound to fusions containing as few as 80 COOH-terminal GHR residues, provided the fusion protein was tyrosine-phosphorylated. By contrast, SOCS-3 binding required tyrosine-phosphorylated GHR membrane-proximal sequences, SOCS-1 binding was tyrosine phosphorylation-independent, and SOCS-6 did not bind the GHR fusion proteins at all. Mutation of GHR's membrane-proximal tyrosine residues 333 and 338 to phenylalanine suppressed the inhibition by SOCS-3, but not by CIS, of GH signaling to STAT5b. SOCS/CIS proteins can thus inhibit GH signaling to STAT5b by three distinct mechanisms, distinguished by their molecular targets within the GHR-JAK2 signaling complex, as exemplified by SOCS-1 (direct JAK2 kinase inhibition), SOCS-3 (inhibition of JAK2 signaling via membrane-proximal GHR tyrosines 333 and 338), and CIS and SOCS-2 (inhibition via membrane-distal tyrosine(s)).

Growth hormone (GH)[1] regulates important physiological processes, including somatic growth and development, carbohydrate and lipid metabolism, and liver metabolic functions, in part through its effects on gene transcription. GH signaling is initiated by hormone-induced receptor dimerization (1), which directly leads trans-phosphorylation associated with activation of the GH receptor (GHR)-associated tyrosine kinase JAK2 (2). JAK2, in turn, phosphorylates GHR on multiple intracellular tyrosine residues, which form docking sites for the transcriptional activator STAT5b and for other GH-activated intracellular signaling molecules (3, 4). STAT5b, as well as the closely related STAT5a, responds directly and repeatedly to the pulsate plasma pattern of pituitary GH secretion (5) that characterizes adult male rats (6, 7). Biochemical and genetic studies strongly suggest that STAT5b is a key intracellular mediator of the effects of plasma GH pulses on several liver-expressed genes, including cytochrome P450 genes expressed in a male-specific manner (6, 8). STAT5b gene disruption leads to an apparent GH pulse insensitivity of liver tissue (55) associated with a decrease in male-characteristic pubertal body growth rate and a loss of male-specific liver gene expression (9, 10). The absence of the male-specific pattern of GH pulse-induced liver gene transcription in adult female rats is linked to the down-regulation of signaling to STAT5b by the female plasma pattern of near-continuous GH exposure (6, 11).

Activation of the GHR by GH initiates negative regulatory pathways important for termination of GH signaling (11–13). One negative regulatory pathway is proposed to involve tyrosine phosphatases, such as SHP-1 and SHP-2 (14), which bind to tyrosine-phosphorylated residues on GHR, JAK2 kinase, and STAT5b via their SH2 domains and may catalyze dephosphorylation leading to inactivation of these signaling molecules (15–19). A second negative regulatory pathway of GH signaling, which is spontaneously activated in liver cells beginning ~40 min after initiation of a GH pulse, requires new protein synthesis (12). This pathway is proposed to involve "immediate early" response genes whose protein products down-regulate signaling to STAT5b by the GHR-JAK2 signaling complex, thereby preventing the activation of additional STAT5b molecules. Precedent for such an inhibitory mechanism is provided by the discovery that cytokine receptor-JAK kinase signal inhibitory molecules, termed SOCS or CIS proteins, are immediate-early gene products that can be induced by a variety of cytokines via a STAT-induced transcriptional mechanism (Refs. 20–22; for review, see Ref. 23). The cytokine signaling inhibitory protein termed SOCS-1 contains an SH2 domain through which it binds to, and thereby inhibits, the JH1 kinase domain of activated, tyrosine-phosphorylated JAK2 kinase (24). By contrast, CIS, a member of the same cytokine inhibitor protein family, is a STAT5-inducible negative feedback regulator that binds to the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3 to block STAT5 phosphorylation and downstream STAT5-dependent transcriptional responses (25, 26). Individual SOCS/CIS mRNAs exhibit unique tissue-specific and time-dependent responses to a broad range of cytokines (22, 27). SOCS/CIS mRNA expression can be induced in liver cells in response to a pulse of GH (28, 29), raising the possibility that one or more of these proteins may contrib-

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[1] The abbreviations used are: GH, growth hormone; GHR, GH receptor; JAK2, Janus tyrosine kinase 2; SOCS, suppressor of cytokine signaling; CIS, cytokine-inducible SH2 protein; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; ntcp, STAT5-activated sodium-dependent taurocholate cotransporter gene; GST, glutathione *S*-transferase; SH2, Src homology 2.

ute to negative feedback regulation of GHR-JAK2 signaling to STAT5b. Indeed, both SOCS-1 and SOCS-3 can inhibit GH-induced STAT5-dependent transcriptional responses in transfected cells (28, 30).

Although GH-activated STATs can transcriptionally activate expression of SOCS/CIS genes, the precise roles of SOCS/CIS proteins in terminating GHR/JAK2 signaling are only partially understood. The present studies were carried out to investigate the mechanisms whereby individual GH-inducible SOCS/CIS proteins inhibit signaling from the GHR-JAK2 receptor-kinase complex to STAT5b. Our findings reveal that SOCS/CIS proteins inhibit GH-stimulated tyrosine phosphorylation of STAT5b and STAT5b-dependent gene transcription by three distinct mechanisms, distinguished by their targets within the GHR-JAK2 signaling complex. GHR tyrosine residues 333 and 338, which are phosphorylated in response to GH stimulation (31) but are not obligatory for GH-stimulated STAT5b activation (32), are presently shown to play a key role in mediating the inhibitory effects of SOCS-3 on GH signaling. These and related findings are discussed in the context of the role of SOCS/CIS proteins in determining the responsiveness of STAT5b to the sex-dependent temporal patterns of plasma GH stimulation.

MATERIALS AND METHODS

Plasmids—Human CIS, SOCS-1, SOCS-2, SOCS-3, and SOCS-6 cDNAs cloned into the expression plasmid pcDNA3 with an NH_2-terminal fused myc tag (27) were provided from Dr. A. Yoshimura, Kurume University (Kurume, Japan). Other designations for these cDNAs include CIS = CIS-1; SOCS-1 = JAB, SSI-1; SOCS-2 = CIS-2, SSI-2; SOCS-3 = CIS-3, SSI-3; and SOCS-6 = CIS-4. Mouse STAT5a and STAT5b cloned into the expression plasmid pME18S (33) were obtained from Dr. A. Mui (DNAX Corp., Palo Alto, CA). Rat GHR cloned into pCDNAI/Amp or into pLM108, a plasmid that contains a metallothionein promoter and an SV40 enhancer (34), and derivatives of pLM108 containing tyrosine to phenylalanine point mutations at residues 333, 338, and both 333 and 338 (32) were kindly provided by Dr. Nils Billestrup (Hagedorn Research Institute, Gentofte, Denmark). Tyrosine to phenylalanine codon replacements were confirmed by DNA sequencing. JAK2 cloned into the expression plasmid pRK5 was obtained from Dr. J. Ihle (St. Jude's, Memphis TN). The STAT5 reporter plasmid 4X-pT109-Luc, which contains four copies of a STAT5 response element from the rat *ntcp* gene (35), was provided by Dr. M. Vore (University of Kentucky, Lexington, KY).

Animals—Male Fischer 344 rats, untreated or hypophysectomized at 8 weeks of age, were purchased from Taconic Farms Inc. (Germantown, NY) and were maintained on a 12-h light-dark cycle. Hypophysectomized rats were administered rat GH (NIDDK-rGH-B-14-SIAFP) at 12.5 μg/100 g body weight by intraperitoneal injection. Longer term GH treatment was by hormone infusion at 2 μg/100 g body weight/h for 1 or 3 days using an Alzet osmotic minipump. Minipumps were implanted subcutaneously as described earlier (6). Rats were killed at different time intervals, livers excised, and stored at −80 °C.

Cell Culture and Transfections—CWSV-1 cells were grown and stimulated with a pulse of GH (500 ng/ml) using methods described previously (36). COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Plasmids and reporter genes were introduced into cells grown in six-well (35 mm diameter) tissue culture plates using the calcium-phosphate method. To obtain similar levels of protein expression, as judged by Western blotting with anti-myc antibody, the following amounts of SOCS/CIS plasmid DNA were used per well: SOCS-1, 0.2 μg; SOCS-3, 1 μg; CIS, SOCS-2, and SOCS-6, 2 μg each. In addition, 0.5 μg of GHR expression plasmid and 0.5 μg of STAT5a or STAT5b expression plasmid were used per well of six-well plates. JAK2 expression plasmid (25 or 70 ng) was used, where indicated. The total amount of transfected DNA was adjusted to 3 μg/well using salmon sperm DNA (Stratagene, La Jolla, CA). At 10–12 h after addition of the DNA-calcium phosphate precipitates, the cells were washed and incubated in Dulbecco's modified Eagle's medium without serum for 36 h. Cells were then stimulated for 30 min with rat GH at a concentration of 500 ng/ml. Whole cell extracts were prepared using lysis buffer (Tropix, Inc.) and stored at −80 °C until ready for STAT5b EMSA and Western blot analysis.

In experiments where STAT5-dependent transcriptional activity was assayed, the STAT5-luciferase reporter plasmid 4X-pT109-Luc (0.4 μg) and the *Renilla* luciferase reporter plasmid pRL-TK (40 ng; Promega Corp., Madison, WI) were cotransfected with expression plasmids encoding STAT5b, GHR (wild-type or tyrosine to phenylalanine mutated, as specified), and a SOCS/CIS protein, as indicated. Cells were stimulated with rat GH (500 ng/ml) added 24 h after the initial addition of the DNA-calcium phosphate precipitates to the cells (see above). Cells were harvested and assayed for both firefly and *Renilla* luciferase activities using a dual luciferase assay kit (Promega, Madison, WI). Normalized luciferase activity values (firefly/*Renilla*) were calculated to adjust for transfection efficiencies between samples. Luciferase and EMSA activity data presented are mean ± S.D. values and are representative of at least two or three independent sets of experiments.

Western Blot and EMSA Analysis—Whole cell extracts (20 μg of protein/lane) were resolved on SDS-polyacrylamide gels, electrotransferred to nitrocellulose and then probed sequentially with anti-STAT and anti-myc antibodies. STAT5b-specific antibody sc-835 is a rabbit polyclonal antibody raised to mouse STAT5b amino acids 711–727 (Santa Cruz Biotechnology). Monoclonal antibody raised to the myc epitope tag (c-myc amino acids 408–439; Santa Cruz Biotechnology sc-40) was used at a dilution of 1/1000. Monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology, Inc. (Lake Placid, NY). EMSA analysis of STAT5b DNA binding activity using a β-casein STAT5 response element probe was carried out using 20 μg of cell extract/well, as described previously (37). Gels were exposed to PhosphorImager plates for quantitation using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software.

RNA Extraction and Northern Blotting—Cell cultures were rinsed twice with ice-cold phosphate-buffered saline; total RNA was extracted from cell cultures and liver samples using guanidium thiocyanate-acid phenol (38). Poly(A) RNA was isolated from the liver RNA samples using oligo(dT)-cellulose columns (Molecular Research Center, Inc., Cincinnati, OH). RNA was fractionated by electrophoresis through agarose-formaldehyde gels and transferred to nylon membranes. Membranes were hybridized at 42 °C overnight with random primed, radiolabeled probes derived from full-length cDNA insert encoding CIS. Membranes were washed at high stringency (0.1× SSC, 0.1% SDS at 42 °C) and exposed to PhosphorImager plates.

Immunoprecipitation and Glutathione S-Transferase (GST) Fusion Binding—Bacteria expressing human GHR cytoplasmic domain-GST fusion proteins cloned into pGEX2TRS and their phosphorylated forms (39) were kindly provided by Dr. Stuart Frank (University of Alabama, Birmingham, AL). Bacteria were grown at 30 °C in LB medium with ampicillin to an $OD_{600\ nm}$ of 0.9, treated with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h to induce expression of the GST-GHR fusion proteins, and lysed by lysozyme treatment in 50 mM Tris-Cl, pH 8, buffer containing 150 mM NaCl, 5 mM EDTA, 4 mM benzamidine, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 mM sodium orthovanadate. Soluble bacterial extracts (12,000 × g × 15 min supernatants) containing GST-GHR fusion proteins were stored at −80 °C. To assay the binding of SOCS/CIS proteins or STAT5b to the GST-GHR fusion proteins, bacterial extracts containing equivalent amounts of each fusion protein bound to glutathione-agarose beads (as determined by anti-GST Western blotting) were incubated for 1.5–2 h at 4 °C with total extracts of COS-1 cells transfected as described in the text (75–100 μg of COS-1 cell extract in 0.1 ml of fusion lysis buffer (Ref. 16)). The beads were washed (16), eluted in SDS gel sample buffer, and then analyzed on a Western blot probed sequentially with anti-STAT5b, anti-myc, and anti-GST antibodies.

JAK2 Phosphorylation—Tyrosine phosphorylation of JAK2 immunoprecipitated from JAK2-transfected COS-1 cells was carried out by anti-phosphotyrosine 4G10 Western blotting as described (16). COS-1 cell extracts (150 μg) were incubated with anti-JAK2 serum (1/1000 dilution) for 3 h at 4 °C. The immunoprecipitates were bound to protein A-Sepharose beads, washed (16), eluted with SDS sample buffer, and then analyzed by Western blotting with anti-phosphotyrosine antibody 4G10. JAK2 recovery was verified by reprobing with anti-JAK2 antibody.

RESULTS

CIS Is Induced by GH Pulse in Rat Liver in Vivo and in Cultured Liver Cells—A physiologic pulse of GH given to hypophysectomized rats stimulates liver STAT5 tyrosine phosphorylation and nuclear translocation within 10 min (6). To determine whether CIS is activated under these conditions of *in vivo* GH treatment, hypophysectomized rats were treated with a pulse of GH, followed by Northern blot analysis of CIS

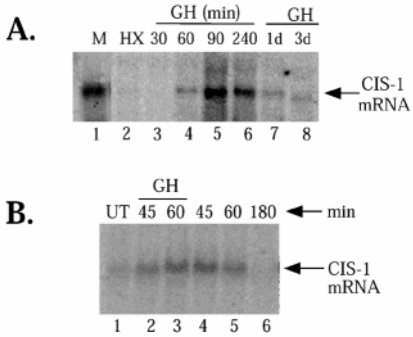

FIG. 1. **Induction of CIS mRNA by GH treatment in rat liver (*panel A*) and in cultured CWSV-1 cells (*panel B*).** *Panel A*, poly(A) RNA was isolated from livers excised from intact (*M*) or hypophysectomized (*HX*) adult male rats treated with a pulse of GH and then killed 30–240 min later (*lanes 3–6*). Poly(A) RNA was also prepared from hypophysectomized male rats treated with GH administered as a continuous infusion using an osmotic minipump for 1 or 3 days (*lanes 7* and *8*). Poly(A) RNA samples (12 μg/lane) were electrophoresed through a formaldehyde gel, transferred to a nylon membrane, hybridized with a cDNA probe for CIS, and visualized by PhosphorImager analysis. *Panel B*, CWSV-1 cells were untreated (*UT*) or were stimulated with GH (500 ng/ml) for 45 or 60 min (*lanes 2* and *3*). Cells treated with GH for 60 min were washed with PBS, fresh medium without GH was then added, and the cells were incubated for an additional 45, 60, or 180 min, as shown (*lanes 4–6*). Northern blot analysis of total CWSV-1 RNA (20 μg/lane) was as described in *panel A*.

mRNA levels. Fig. 1*A* shows that hypophysectomy substantially decreases the endogenous CIS mRNA signal. Moreover, CIS mRNA was strongly induced by a single pulse of GH. The increase in CIS mRNA was first detected at 60 min, was maximal by 90 min, and was still detectable at 4 h. By contrast, GH given as a continuous infusion, a pattern that mimics the female plasma GH pattern, activated CIS gene expression at a lower level, which continued for several days (Fig. 1*A*, *lanes 7* and *8*). In experiments carried out using the GH-responsive rat liver cell line CWSV-1 (36), GH given in the form of a 1-h pulse induced CIS mRNA within 45 min (Fig. 1*B*). The induced CIS mRNA was maintained for a full 1 h after removal of the GH stimulus (*lanes 4* and *5*) but decayed substantially within 3 h (*lane 6*). This time course correlates with the time course for termination of GHR-JAK2 signaling to STAT5b, which becomes evident by ~40 min after GH stimulation (12). Moreover, the decay of CIS mRNA expression within 3 h after termination of the GH pulse corresponds to the time required to regain GH pulse-responsiveness of STAT5b activation in these cells (36). GH-stimulated expression of other SOCS/CIS family RNAs has been described in a mouse liver model (28).

SOCS/CIS Proteins Inhibit GH Activation of STAT5—To study the functional role of SOCS/CIS proteins in the termination of GH signaling to STAT5, we utilized a COS-1 cell transfection model, where the effects of individual SOCS/CIS proteins on STAT5b activation and their functional interactions with GHR and JAK2 kinase could be evaluated. These studies utilized expression plasmids encoding five SOCS/CIS proteins, each fused to an amino-terminal myc epitope tag (27). COS-1 cells were transiently transfected with expression plasmids encoding GHR and STAT5b, in the presence or absence of a SOCS/CIS expression plasmid. Treatment of the cells with a pulse of GH for 30 min strongly activated STAT5b DNA binding activity, as shown by EMSA using a DNA probe containing a STAT5 response element derived from the rat β-casein gene (Fig. 2*A*, *lane 2 versus lane 1*). Co-expression of CIS or SOCS-2 partially inhibited STAT5b activation (Fig. 2*A*, *lanes 4*, *5*, *7*, and *8 versus lane 2*; Fig. 2*C*), while SOCS-3 and SOCS-1 fully blocked GH-stimulated STAT5b activation (Fig. 2*A*, *lanes 10*, *11*, *13*, and *14*). STAT5b activation was not inhibited in COS-1

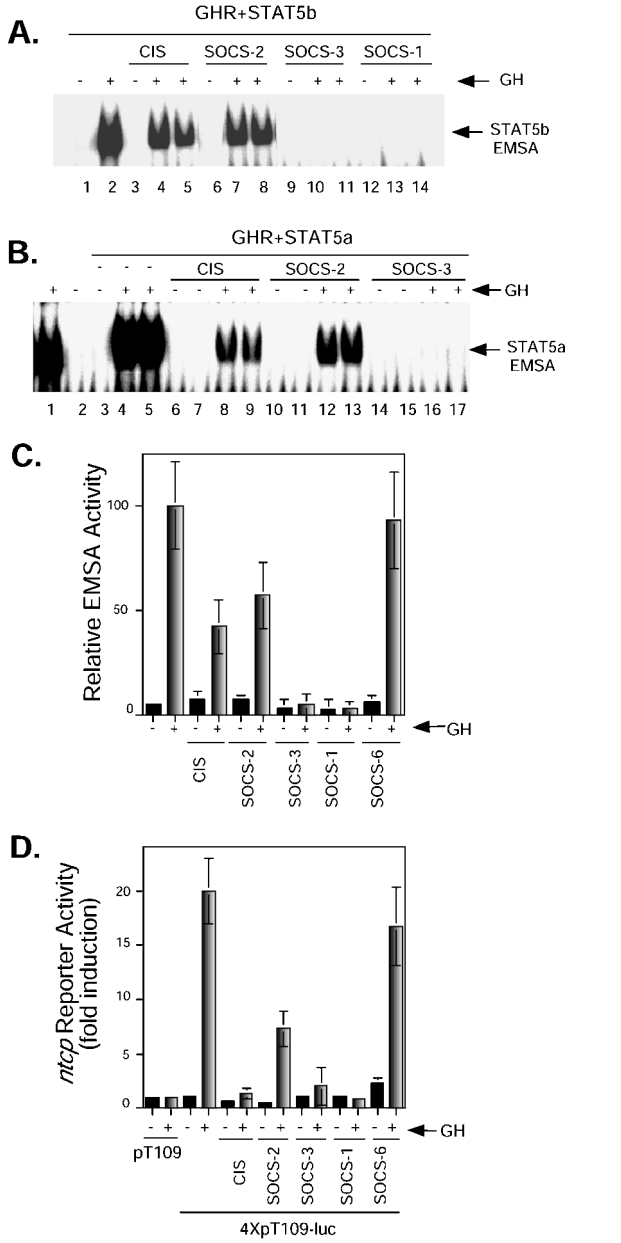

FIG. 2. **SOCS protein inhibition of STAT5b activation in COS-1 cells.** COS-1 cells were transiently transfected with cDNAs expressing GHR and either STAT5b (*panels A*, *C*, and *D*) or STAT5a (*panel B*) in the presence of cDNAs encoding the indicated SOCS/CIS proteins. Cells were treated with GH for 30 min, washed with cold buffer and cell extracts prepared (*panels A–C*). In *panel D*, cells were cotransfected with the STAT5 luciferase reporter gene 4X-pT109-Luc, or the empty vector pT109, as indicated, and were stimulated with GH for 24 h prior to preparation of cell extracts. *Panel A* presents an EMSA using β-casein probe, which shows that GH-inducible STAT5b DNA binding activity (*lane 2*) is fully blocked in the presence of SOCS-3 or SOCS-1 (*lanes 10*, *11*, *13*, and *14*) and is partially blocked in cells expressing CIS or SOCS-2 (*lanes 4*, *5*, *7*, and *8*). *Panel B* presents an EMSA analysis similar to that shown in *panel A* for cells transfected with STAT5a in place of STAT5b. Shown in *lane 1* is an EMSA of GH-activated STAT5b, which migrates faster than the STAT5a-DNA complex seen in *lanes 4* and *5*. *Panel C* presents STAT5b EMSA data obtained from a series of experiments similar to that shown in *panel A* (mean ± S.D., $n \geq 5$). GH-stimulated STAT5b EMSA activity in the presence of the indicated SOCS/CIS proteins is graphed as a percentage of the activity obtained in the absence of SOCS/CIS protein expression. *Panel D* presents the effects of the indicated SOCS/CIS proteins on STAT5b-stimulated ntcp reporter activity, determined using a dual luciferase assay method. Normalized luciferase activity are graphed as -fold induction values relative to ntcp reporter activity without GH stimulation (mean ± S.D., $n \geq 4$).

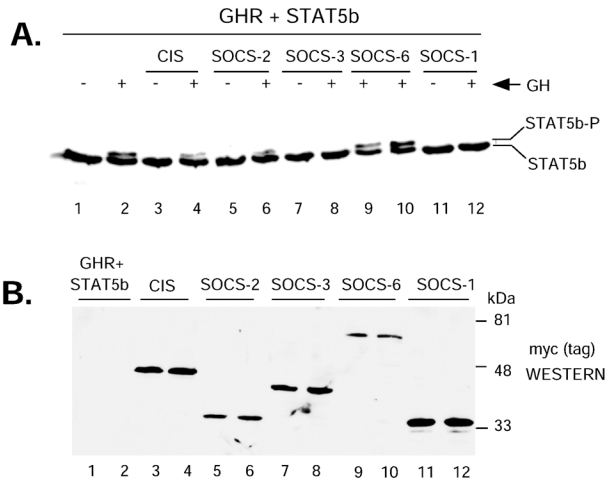

FIG. 3. **SOCS/CIS inhibition of STAT5b tyrosine phosphorylation.** *Panel A* presents a STAT5b Western blot of COS-1 cells transfected with GHR, STAT5b, and the indicated SOCS/CIS proteins and treated with GH as described in Fig. 2*A*. The major STAT5b bands resolved on the blot shown were previously identified as unphosphorylated STAT5b (*lane 1*), and tyrosine/serine-diphosphorylated STAT5b (*upper band* in *lane 2*; *band* marked *STAT5b-P*) (36). Only the tyrosine-phosphorylated form of STAT5b binds DNA when assayed by EMSA using the β-casein probe. *Panel B*, the blot shown in *panel A* was reprobed with anti-myc antibody to visualize each of the transfected SOCS/CIS proteins. Migrations of protein size markers were as indicated on the *right*.

cells expressing SOCS-6 (Fig. 2*C*). SOCS/CIS expression led to a corresponding pattern of inhibition of STAT5b transcriptional activity, which was assayed using a highly responsive STAT5b-luciferase reporter gene containing four copies of a STAT5b binding site derived from the liver-expressed gene *ntcp* (35). GH treatment activated the ntcp reporter ~20-fold but had no effect on the empty vector pT109 (Fig. 2*D*). SOCS-1 and SOCS-3 were highly inhibitory toward GH induction of ntcp reporter activity. By contrast, SOCS-2 was partially inhibitory and SOCS-6 was non-inhibitory, in agreement with the effects of these SOCS proteins on STAT5b EMSA activity. CIS inhibited GH induction of ntcp reporter gene activity to a greater extent than would be anticipated based on the degree of STAT5b EMSA inhibition (*e.g.* Fig. 2*D versus* Fig. 2*C*); this differential effect was observed over a range of CIS plasmid levels (data not shown).

The inhibitory effects of the SOCS/CIS proteins were also seen when GH activation of STAT5a was evaluated. Thus, SOCS-1 (data not shown) and SOCS-3 were highly inhibitory to STAT5a EMSA activity (Fig. 2*B*, *lanes 16* and *17 versus lanes 4* and *5*), while CIS and SOCS-2 were partially inhibitory (*lanes 8* and *9* and *lanes 12* and *13*, respectively). STAT5a is closely related to STAT5b (>90% identical coding sequence) and responds to GH in a manner similar to STAT5b but exhibits differences from STAT5b with respect to its DNA binding specificity (40, 41) and its role in the sex-dependent and gene-specific effects that GH has on the liver (10).

GH activation of STAT5b can be monitored by the conversion of STAT5b to a slower migrating form, which is readily detected on a Western blot of cell extracts probed with STAT5b antibody (Fig. 3*A*, *lane 2 versus lane 1*) and was previously identified as a tyrosine-phosphorylated form (36, 37). STAT5b tyrosine phosphorylation was strongly inhibited in COS-1 cells expressing SOCS-1 or SOCS-3 (Fig. 3*A*, *lanes 8* and *12 versus lane 2*) and was partially inhibited in cells expressing CIS or SOCS-2 (compare ratio of upper *versus* lower STAT5b band in *lanes 4* and *6 versus lane 2*). No affect of SOCS-6 on STAT5b phosphorylation was seen (*lanes 9* and *10*). Reprobing the Western blot with antibody to the myc tag confirmed the expression of each of the SOCS/CIS proteins (Fig. 3*B*). Together, these findings demonstrate that four of the five SOCS/CIS proteins examined can inhibit GH-induced STAT5b activation and STAT5b-dependent transcriptional activity, and that SOCS-1 and SOCS-3 are more potent inhibitors than CIS and SOCS-2.

SOCS/CIS Proteins Inhibit JAK2 Phosphorylation by Two Mechanisms—While SOCS-1 has been implicated as a direct inhibitor of JAK kinases (24), the mechanisms for the signal inhibitory actions of other SOCS/CIS proteins, in particular as they relate to GH signaling, are unknown. Given the critical importance of JAK2 kinase in GH/STAT5 signal transduction (2), we examined the effects of each of the SOCS/CIS proteins on JAK2 activation, which was evaluated by monitoring JAK2 tyrosine phosphorylation. Although JAK2 is expressed endogenously in COS-1 cells at a level sufficient to support GH-stimulated activation of transfected STAT5 (*e.g.* Fig. 2), phosphorylated JAK2 is present at a level that is just barely detectable when analyzed by immunoprecipition followed by anti-phosphotyrosine Western blotting (Fig. 4*A*, *lane 2 versus lane 1*). By contrast, tyrosine-phosphorylated JAK2 could readily be detected in cells transfected with a low level of exogenous JAK2 (25 ng of plasmid) (Fig. 4*A*, *lane 8*). JAK2 phosphorylation in these cells was constitutive (*i.e.* GH-independent) (*lane 7 versus lane 8*). Interestingly, this constitutive JAK2 phosphorylation was only observed when GHR was co-expressed (*lanes 7* and *8 versus lanes 3* and *4*). This finding suggests that, in cells where JAK2 is overexpressed, the co-transfected GHR dimerizes and becomes activated in a ligand-independent manner, which in turn leads to JAK2 kinase activation and *trans*-phosphorylation. As expected, the constitutive tyrosine phosphorylation of JAK2 results in a substantial GH-independent activation of STAT5b (Fig. 4*C*, *lanes 1* and *2 versus lanes 3* and *4*; *cf.* Fig. 2*A*, *lanes 1* and *2*).

We next examined the effects of SOCS/CIS expression on JAK2 tyrosine phosphorylation. Fig. 4*B* shows that CIS and SOCS-2 both inhibited JAK2 phosphorylation, albeit not completely (*lanes 5–10 versus lanes 1–4*), while SOCS-1 and SOCS-3 were fully inhibitory (*lanes 11–18*). This, in turn, resulted in a substantial inhibition of both basal and GH-stimulated STAT5b EMSA activity, consistent with the established requirement of active, tyrosine-phosphorylated JAK2 for STAT5b tyrosine phosphorylation and DNA binding (Fig. 4*C*; note strong intensity of EMSA signal in *lanes 1–4 versus* SOCS/CIS-inhibited signals in *lanes 5–20*). Two potential mechanisms for the observed inhibition of JAK2 phosphorylation by the four SOCS/CIS proteins are: 1) CIS, SOCS-2 and/or SOCS-3 may block STAT5 activation by inhibiting JAK2 activity directly, as has been demonstrated for SOCS-1; alternatively, 2) the observed inhibitions may result from SOCS/CIS binding to the GHR/JAK2 complex in a manner that interferes with the receptor dimerization step or other events required for JAK2 activation. These two possibilities were distinguished in experiments where JAK2 expression plasmid was transfected into the cells at a level that was found to be sufficient for constitutive JAK2 tyrosine phosphorylation in the *absence* of GHR (70 ng of JAK2 plasmid). Under these conditions, SOCS-1 strongly inhibited JAK2 phosphorylation (Fig. 4*D*, *lanes 9* and *10 versus lanes 1* and *2*), while the other SOCS/CIS proteins exhibited much less or no inhibitory activity (Fig. 4*D*, *lanes 3–8*, *cf.* Fig. 4*B*). Thus, in contrast to SOCS-1, the inhibition of JAK2 phosphorylation by CIS, SOCS-2, and SOCS-3 is dependent on the presence of GHR and thus may proceed via an indirect mechanism.

Individual SOCS/CIS Proteins Exhibit Different Require-

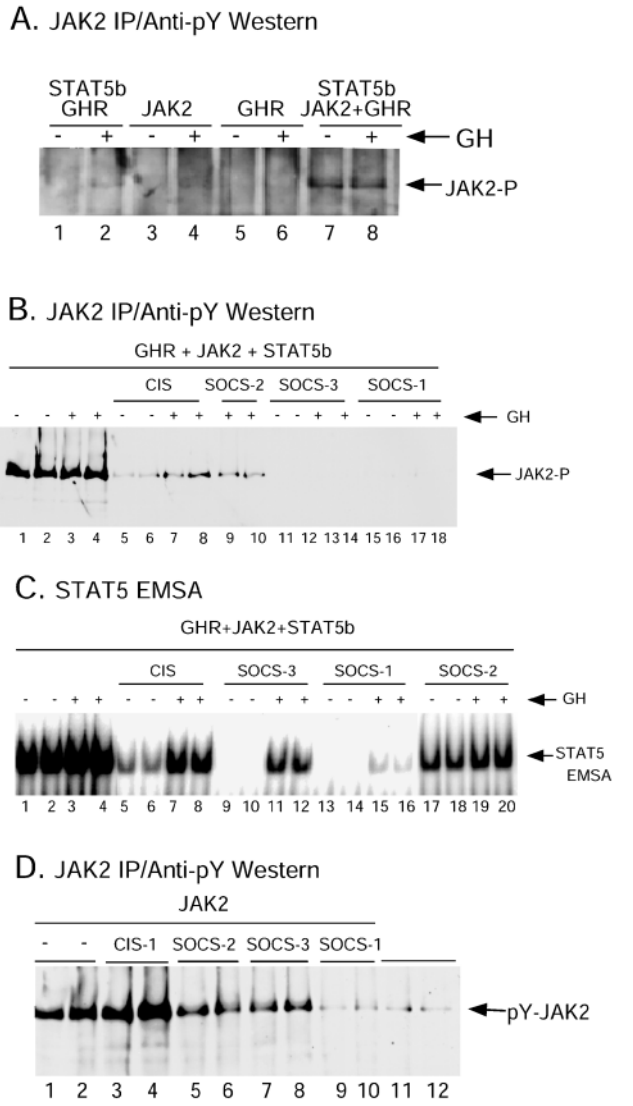

FIG. 4. **Impact of SOCS/CIS proteins on JAK2 tyrosine phosphorylation.** Shown in *panels A*, *B*, and *D* are anti-phosphotyrosine Western blots of anti-JAK2 immunoprecipitated COS-1 cell extracts. COS-1 cells were transfected with expression plasmids for JAK2, GHR, STAT5b, and/or each of the indicated SOCS/CIS proteins. Cells were stimulated with GH, and extracts were immunoprecipitated with anti-JAK2 antiserum, electrophoresed, and blotted with anti-phosphotyrosine antibody 4G10. *Panel A* shows phosphorylation of endogenous COS-1 cell JAK2 that is just barely detectable (*lane 2*) but can be greatly increased (*lane 8*) by transfection of exogenous JAK2 (25 ng/well). Under these conditions of transfection, JAK2 phosphorylation does not require GH treatment (*lane 7*) but is dependent on GHR cotransfection (*cf. lanes 3* and *4*). *Panel B* shows the effects of SOCS/CIS expression on the tyrosine phosphorylation of JAK2 in cells transfected with JAK2 (25 ng/well) together with GHR and STAT5b. *Panel C* presents STAT5 EMSA analysis of the same series of samples shown in *panel B*. *Panel D* shows the effects of SOCS/CIS expression on tyrosine phosphorylation of JAK2 in cells transfected with higher levels of JAK2 (70 ng/well), which leads to constitutive JAK2 activation (*lanes 1* and *2*) even in the absence of GHR cotransfection. Under these conditions, SOCS-1 but not the other SOCS/CIS proteins decreased JAK2 tyrosine phosphorylation down to the low basal level seen in COS-1 cells without JAK2 transfection (*lanes 11* and *12*).

ments for Binding to GHR Cytoplasmic Domain—We next examined whether any of the SOCS/CIS proteins can interact with the COOH-terminal, cytoplasmic domain of GHR. Precedent for this possibility is provided by the report that CIS binds the tyrosine-phosphorylated cytoplasmic tail of the erythropoietin receptor to inhibit signaling to STAT5 (25, 26, 42). Fusion proteins containing GST linked to various segments of the

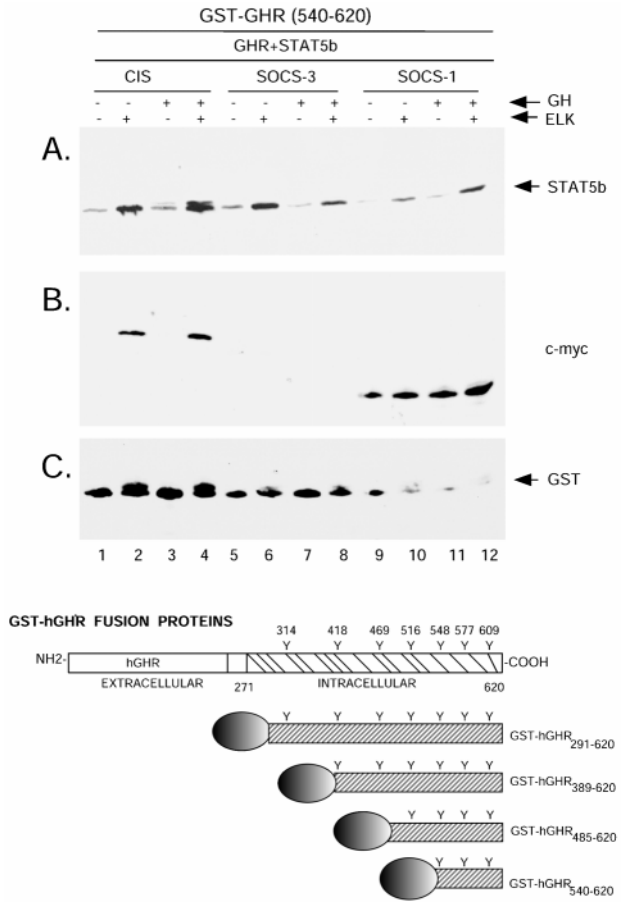

FIG. 5. **Binding of SOCS/CIS proteins to GST-GHR-(540–620).** COS-1 cells were cotransfected with GHR, STAT5b and the indicated SOCS/CIS plasmids and then stimulated with GH for 30 min, as indicated. Cell extracts were incubated for 2 h at 4 °C with glutathione-agarose beads prebound with the fusion protein GST-GHR-(540–620), expressed in *E. coli* with or without Elk kinase-stimulated tyrosine phosphorylation of the fusion protein, as indicated. Washed glutathione-agarose beads were eluted in SDS gel sample buffer and analyzed on a Western blot probed sequentially with anti-STAT5b (*A*), anti-myc (*B*), and anti-GST antibody (*C*). The apparent recovery of GST-GHR fusion protein was low in the SOCS-1-bound samples (*lanes 9–12*). (Also see Fig. 6*D*, *lanes 11* and *12*.) Shown at the *bottom* is a schematic of human GHR and each of the four GST-GHR fusion proteins used in Figs. 5 and 6. Tyrosine residues (*Y*) are specified using the human GHR numbering system, where residue 620 is the COOH-terminal amino acid, in contrast to rat GHR, where the corresponding residue is numbered 638. Similary, human GHR tyrosine 314 corresponds to rat GHR tyrosine 333. Rat GHR tyrosine 338 is replaced by a histidine residue in the human receptor protein. The other six human GHR tyrosine residues shown (*Y418* to *Y609*) are conserved in rat and other species.

COOH-terminal region of GHR (Fig. 5, *bottom*) (39) were expressed in bacteria under conditions where the fusion protein was unmodified or, alternatively, was tyrosine-phosphorylated by coexpression of the Elk tyrosine kinase (43). Lysates of COS-1 cells, transfected with expression plasmids encoding GHR, STAT5b, and the individual SOCS/CIS proteins, were incubated with the GST-GHR fusion proteins immobilized on glutathione-Sepharose beads. The GST-GHR-bound proteins were then analyzed by Western blot for the presence of bound STAT5b or SOCS/CIS protein, detected using anti-STAT5b and anti-myc antibodies, respectively. Fig. 5*B* shows that CIS bound to a GST-GHR fusion containing ~80 amino acids from the COOH terminus of GHR (residues 540–620), but only when the GHR sequence was tyrosine-phosphorylated (*lanes 2* and *4 versus lanes 1* and *3*). By contrast, SOCS-1 bound to GST-GHR-(540–620) even in the absence of GHR tyrosine phosphorylation (*lanes 9–12*), while SOCS-3 did not bind at all (*lanes 5–8*).

Binding of CIS and SOCS-1 to the GHR fusion protein was not dependent on GH stimulation (*lane 2 versus lane 4* and *lane 10 versus lane 12*), indicating that a GH-induced SOCS/CIS protein phosphorylation is not required for receptor binding. Reprobing of the Western blot with antibody to GST confirmed the recovery of GST fusion protein in the SOCS-3 samples (*panel C*). In addition, reprobing with STAT5b antibody revealed a preferential binding of STAT5b to the tyrosine-phosphorylated fusion protein in all of the samples, in agreement with previous studies (39). Phosphorylation of STAT5b in response to GH was more completely inhibited by expression of SOCS-1 or SOCS-3 (*panel A*, *lanes 7*, *8*, *11*, and *12*) than CIS (*lanes 3* and *4*; upper band of STAT5b doublet, and data not shown), in agreement with Fig. 3*A*.

Further investigation revealed that SOCS-3 could bind to the GHR cytoplasmic domain, but only when the receptor fragment was lengthened to include all of the cytoplasmic domain's tyrosine residues, GST-GHR-(291–620) (Fig. 6*A*, *lane 3 versus lanes 4–6*). Binding was only detected when the GHR fragment was tyrosine-phosphorylated (*lane 3 versus lane 2*). By contrast, SOCS-1 bound with similar efficiency to GHR fusion constructs ranging in length from GHR residues 540–620 to 291–620; in each case this binding was not significantly affected by tyrosine phosphorylation of the GHR fusion protein (*lanes 8–12* and data not shown). SOCS-2 bound to GST-GHR-(291–620) and to GST-GHR-(540–620) only when the fusion proteins were tyrosine-phosphorylated (Fig. 6*C*, *lanes 5* and *6*, and data not shown). SOCS-6 did not bind to any of the GHR fusion proteins (Fig. 6*C*, *lanes 9* and *10*, and data not shown), consistent with the absence of a SOCS-6 inhibitory effect on STAT5b activation (Fig. 2, *C* and *D*). In control experiments, none of the SOCS/CIS proteins bound to GST alone, demonstrating a specific requirement for the GHR COOH-terminal sequences for the observed binding interactions (data not shown). Reprobing the blots with anti-GST antibody verified the size lengths of the individual GST-GHR fusion proteins and the recovery of fusion protein in all of the samples (Fig. 6, *B* and *D*).

GHR Tyrosines 333/338 Contribute Functionally to SOCS-3 Inhibition—The finding that SOCS-3 binds specifically to tyrosine-phosphorylated GST-GHR-(291–620), but not to tyrosine-phosphorylated GST-GHR-(389–620) (Fig. 6*A*), suggests that the membrane-proximal tyrosine residues 333 and/or 338 of GHR may be specifically required for the inhibitory effects of SOCS-3 on STAT5b activation and transcriptional activity. Tyrosines 333 and 338 are phosphorylated in GH-stimulated cells (31), but are not obligatory for GH stimulation of STAT5 transcriptional activity (32). To test this hypothesis, COS-1 cells were transfected with wild-type GHR or with GHR-Y333F,Y338F, which contains site-specific mutations of tyrosines 333 and 338 to phenylalanine (32). Wild-type and mutated GHR both supported GH-stimulated STAT5b activation and STAT5b-dependent ntcp reporter activity in a very similar manner (Fig. 7, legend). Co-expression of SOCS-3 with wild-type GHR led to dose-dependent inhibition of both GH-stimulated activities (Fig. 7*A*, *left*, and data not shown), consistent with Fig. 2. By contrast, with GHR-Y333F,Y338F, low concentrations of SOCS-3 expression plasmid (0–120 ng) were minimally inhibitory to STAT5 signaling (STAT5b EMSA activity; data not shown) or were non-inhibitory (STAT5b-induced ntcp reporter activity; Fig. 7*A*, *right*). This decreased effectiveness of SOCS-3 expression plasmid at inhibiting GH signaling supported by GHR-Y333F,Y338F is not due to inefficient SOCS-3 expression, as revealed by Western blot analysis of the expressed SOCS-3 protein (Fig. 7*A*, *bottom*). Together, these findings demonstrate a requirement for tyrosines 333 and/or 338

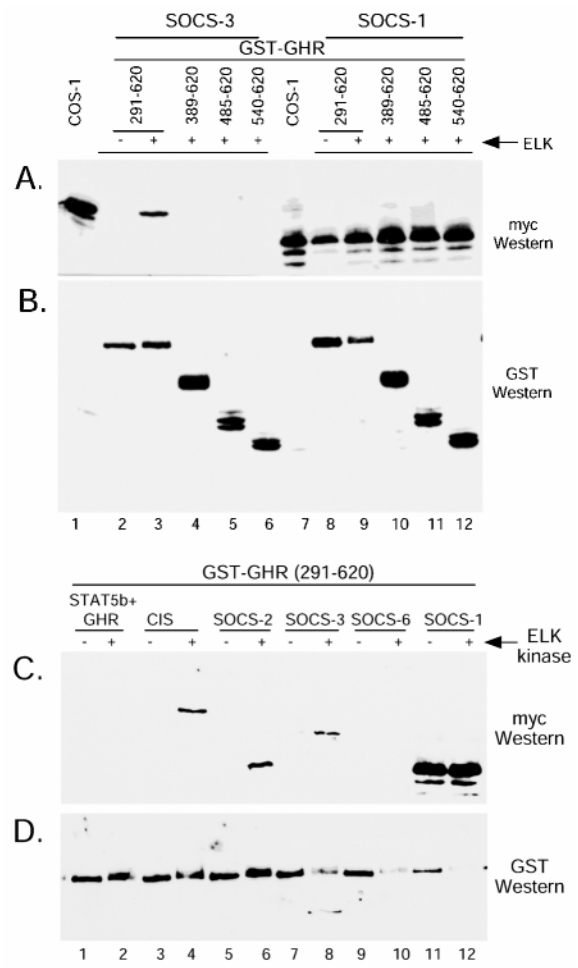

FIG. 6. **Association of SOCS-3 and SOCS-1 with GST-GHR fusion proteins.** *Panel A*, extracts from COS-1 cells transfected with the indicated SOCS/CIS proteins, but in the absence of GHR and STAT5b co-transfection, were incubated with glutathione-agarose-bound GST-GHR fusion proteins as described in Fig. 5. Elution of the bound COS-1 proteins, followed by sequential immunoblotting with anti-myc (*panels A* and *C*) and anti-GST (*panels B* and *D*) antibody for detection of SOCS/CIS proteins and GST-GHR fusion proteins, respectively, were as described in Fig. 5. SOCS-3 binding to GST-GHR required the presence of tyrosine-phosphorylated, GHR membrane-proximal sequences between residues 291 and 389 (*panel A*, *lane 3*), whereas SOCS-1 binding was already maximal with the shortest construct, GST-GHR-(540–620) and was independent of GHR tyrosine phosphorylation (*panel A*, *lanes 8–12*). *Lanes 1* and *7* of *panel A* show COS-1 cell extracts transfected with SOCS-3 and SOCS-1, respectively. CIS and SOCS-2 binding to GST-GHR also required tyrosine phosphorylation of the fusion protein (*panel C*, *lanes 3–6*). Strong binding of SOCS-1 to GST-GHR-(291–620) was evident, despite the low apparent recovery of GST fusion protein (*panel D*, *lanes 11* and *12*).

for the inhibition associated with high affinity binding of SOCS-3 to GHR. By contrast, CIS was equally effective as an inhibitor of ntcp reporter activity supported by wild-type GHR or by GHR-Y333F,Y338F (Fig. 7*B*). This is consistent with our observation (Fig. 5) that CIS binds to membrane-distal phosphorylated tyrosine(s) of GHR. Interestingly, at higher levels of transfected SOCS-3, substantial inhibition of GHR-Y333F, Y338F-dependent activity was observed (Fig. 7*A*). At these higher levels, SOCS-3 may inhibit STAT5 activation by binding to targets distinct from tyrosines 333 and 338.

Finally, we examined the role of tyrosines 333 and 338 individually in supporting SOCS-3 inhibition of STAT5 activity. As seen in Fig. 7*C*, SOCS-3 was inhibitory to STAT5 activation supported by GHRs with single tyrosine to phenylalanine mutations, GHR-Y333F and GHR-Y338F. Interestingly, partial

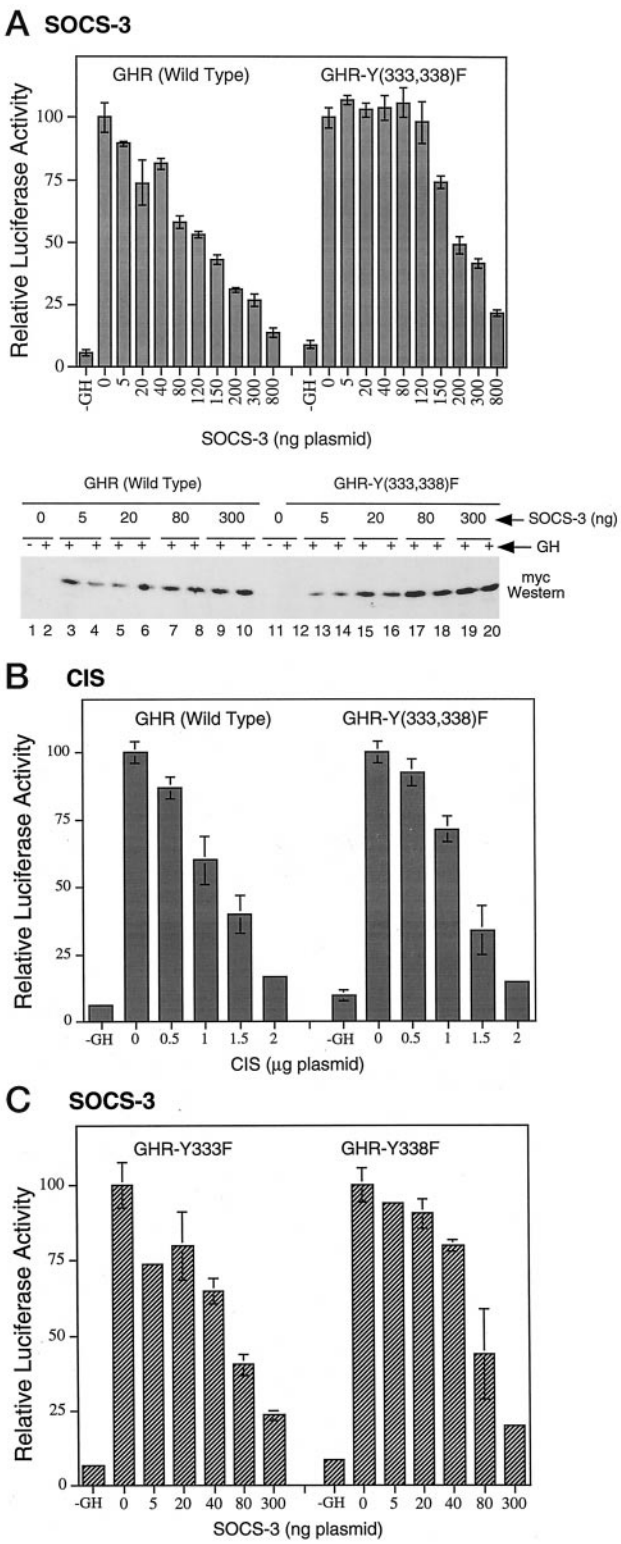

FIG. 7. **Impact of GHR tyrosines 333 and 338 on SOCS/CIS inhibition of STAT5b-stimulated transcription.** COS-1 cells were transfected with wild-type GHR or with GHR containing tyrosine to phenylalanine point mutations at residues 333 and/or 338, as indicated. Cells were co-transfected with STAT5b and the reporter gene 4x-pT109-Luc in the presence of SOCS-3 expression plasmid (0–800 ng) (*panels A* and *C*) or CIS expression plasmid (0–2 μg) (*panel B*). Cells were stimulated with GH for 24 h, and normalized luciferase reporter activity was assayed as described under "Materials and Methods." GHR-Y333F,Y338F is shown to be resistant to the inhibitory effects of SOCS-3 (0–120 ng; *panel A*) but not that of CIS (*panel B*). Partial resistance to the inhibitory effects of SOCS-3 (0–40 ng) in the case of GHR-Y338F is suggested by *panel C*. The *lower portion* of *panel A* shows a Western blot probed with antibodies to myc to visualize SOCS-3 expression in samples corresponding to a subset of those samples shown in the *upper portion* of *panel A*. Data shown are based on two or three independent series of transfections and in most cases represent mean ± S.D. values for $n \geq 4$ samples. Representative normalized luciferase activity values for GH-stimulated samples in the absence of SOCS/CIS expression were 20.0 for wild-type GHR, 18.0 for GHR-Y333F,Y338F, 20.5 for GHR-Y333F, and 16.6 for GHR-Y338F; thus, the GHR tyrosine to phenylalanine substitutions had little impact on the transcriptional activity of STAT5b.

protection from SOCS-3 inhibition at low concentrations of plasmid DNA (≤20 ng) could be discerned in the case of the GHR-Y338F mutant. These findings suggest that GHR tyrosines 333 and 338, when phosphorylated in response to GH stimulation, may serve as alternative sites for SOCS-3 binding and inhibition of receptor activity.

DISCUSSION

SOCS/CIS genes encode a novel family of inhibitors of cytokine and hormone signaling that can be rapidly induced following the initiation of signaling from a cell surface-bound receptor-JAK kinase complex. SOCS/CIS proteins contain a COOH-terminal "SOCS box" that may target these proteins to proteasomal degradation (44), and a central SH2 domain that mediates SOCS/CIS binding to phosphotyrosine residues in target proteins. Established SOCS/CIS targets include JAK2 kinase in the case of SOCS-1 (also termed JAB) (24) and the intracellular domain of the erythropoietin receptor for SOCS-3 (42). The present investigation of the inhibitory activity of five SOCS/CIS proteins toward GH-stimulated signaling to STAT5a and to STAT5b established SOCS-1 and SOCS-3 to be highly potent inhibitors of GH signaling. CIS and SOCS-2 were somewhat weaker inhibitors when expressed at similar protein levels, and SOCS-6 was non-inhibitory. The lack of inhibition of GH-dependent gene transcription by CIS and SOCS-2 under the conditions of transfection used in an earlier study (28) could be explained by the less efficient expression (perhaps reflecting greater instability) of those SOCS/CIS proteins compared with SOCS-1 and SOCS-3 seen in our studies (data not shown). Three distinct mechanisms of SOCS/CIS inhibition of GH signaling were identified, with SOCS-1 acting at the level of JAK2 tyrosine kinase, SOCS-3 acting at the level of the membrane-proximal tyrosine residues 333 and 338 of GHR, and CIS and SOCS-2 acting via membrane-distal GHR tyrosine residue(s). This demonstration that individual SOCS/CIS proteins interact with distinct molecular targets within the GHR-JAK2 signaling complex, together with the observation of distinct kinetics of induction and decay for individual SOCS/CIS proteins in GH-stimulated liver cells (Fig. 1 and Refs. 28 and 29), suggest that each of the GH-inducible SOCS/CIS genes may play a unique role in termination of hepatic GH signaling.

A key feature shared by all SOCS/CIS proteins is the central SH2 domain (45), which enables these proteins to directly bind to tyrosine-phosphorylated signaling molecules. In the case of SOCS-1, JAK2 tyrosine kinase has been identified as the primary target (20, 21), a finding that is consistent with the strong inhibition by SOCS-1, but not the other SOCS/CIS proteins, of JAK2 tyrosine phosphorylation seen in the absence of GHR (Fig. 4*D*). SOCS-1 inhibition of JAK2 kinase activity requires binding interactions between SOCS-1's SH2 domain and tyrosine 1007 within JAK2's kinase activation loop (24). The inhibition of JAK2 activity additionally requires a 13-residue "kinase inhibitory peptide" that is immediately NH_2-terminal to SOCS-1's extended SH2 domain, and is proposed to function as an inhibitory, non-phosphorylatable pseudosubstrate of JAK2 (24, 46). Our finding that SOCS-1 can bind to the 80 COOH-terminal cytoplasmic residues of GHR, even in the absence of

tyrosine phosphorylation (Figs. 5 and 6), suggests that SOCS-1 may be associated with unstimulated receptor molecules, and points to an additional mechanism whereby SOCS-1 can interact with its target signaling molecules. Conceivably, the binding of SOCS-1 to these GHR sequences may serve to correctly orient SOCS-1 or perhaps increase its affinity for the receptor-kinase signaling complex.

CIS, SOCS-2 and SOCS-3 were each found to bind specifically to tyrosine-phosphorylated GHR cytoplasmic tail sequences. This binding was observed in the absence of GH stimulation (Fig. 6), and thus does not require GH-induced tyrosine phosphorylation of the SOCS/CIS proteins or of any other signaling molecules. These results are most readily explained by direct binding interactions between phosphotyrosine residue(s) on GHR and the SH2 domains of CIS, SOCS-2, and SOCS-3. Tyrosine phosphorylation of SOCS-3 occurs in interleukin-2-stimulated cells (47); however, we were unable to detect basal or GH-inducible tyrosine phosphorylation of any of the SOCS/CIS proteins examined in our studies (data not shown).

In the case of CIS and SOCS-2, but not SOCS-3, GHR residues 540–620 were sufficient for SOCS/CIS binding to the receptor (Figs. 5 and 6). GH stimulates phosphorylation of two tyrosines within this region of GHR (rat GHR tyrosines 566 and 627; equivalent to human GHR residues 548 and 609; Fig. 5), both of which provide functional docking sites for STAT5 (48). Accordingly, the inhibition by CIS and SOCS-2 of GHR-JAK2 signaling to STAT5 (Fig. 2) could result from direct competition between STAT5 and either CIS or SOCS-2 for tyrosine-phosphorylated binding sites on GHR. Alternatively, the inhibition of GH signaling by CIS or SOCS-2 could involve SOCS/CIS protein ubiquitination followed by degradation of the SOCS/CIS-bound GHR-JAK2 complex. This could result in a rapid turnover of the SOCS/CIS proteins, as proposed for CIS in the case of the erythropoietin receptor (42). GH also stimulates phosphorylation of several upstream GHR tyrosines, including residues 333, 338, 487, and 537. These tyrosines can support GH signaling via STAT5 to varying extents (32, 48–50), and may serve as additional targets for CIS or SOCS-2. The somewhat weaker inhibition of GH signaling to STAT5 seen with CIS and SOCS-2, compared with SOCS-1 and SOCS-3, could in part be a reflection of this multiplicity of STAT5 binding sites if, as seems likely, CIS and SOCS-2 only bind to a subset of these sites.

SOCS-3 was unique among the five SOCS/CIS proteins in terms of its requirement for membrane-proximal phosphorylated tyrosines for GHR binding (Fig. 6). The GHR region suggested to be involved in SOCS-3 binding, residues 291–388, contains either one (human GHR tyrosine 314) or two conserved tyrosine residues (GHR tyrosines 333 and 338 in rat and other species), which undergo GH-induced phosphorylation (31). Tyrosines 333 and 338 are required for GH-stimulated lipogenesis and protein synthesis (32), suggesting that SOCS-3 could play a modulatory role in these cellular responses to GH. Mutation of both membrane-proximal tyrosines to phenylalanine blocked the inhibition of STAT5b phosphorylation and STAT5b-dependent transcriptional activity seen at low concentrations of SOCS-3, but not the inhibition effected by CIS (Fig. 7), supporting our proposal that either or perhaps both of these tyrosines (Fig. 7*C*) serve as key targets for inhibition by SOCS-3. At higher SOCS-3 levels, inhibition of GH signaling was observed with both the wild-type and the Y333F,Y338F mutant GHR, suggesting that additional inhibitory targets and mechanisms are utilized at high intracellular SOCS-3 concentrations. These additional mechanisms may include SOCS-3 binding to COOH-terminal GHR phosphotyrosine residues or perhaps direct JAK2 inhibition by SOCS-3, even in the absence of its GHR docking site (see below).

Several possibilities can be considered for the mechanism of GH signaling inhibition by SOCS-3. Binding of SOCS-3 to phosphorylated tyrosines 333 and 338 might directly compete for STAT5 binding; however, this seems unlikely, insofar as these tyrosines are not the major docking sites for STAT5 (48). Moreover, this mechanism does not account for the observed inhibition by SOCS-3 of JAK2 tyrosine phosphorylation in a GHR-dependent manner (Fig. 4). A second possibility is that SOCS-3, once bound to GHR, could facilitate ubiquitination leading to degradation of the GHR-JAK2 complex, either directly, as discussed above for CIS, or indirectly via elongins B and C (44). A third possible mechanism is suggested by our observation that SOCS-3 strongly inhibits JAK2 phosphorylation in the presence but not in the absence of GHR. This contrasts to the strong intrinsic inhibition of JAK2 activity by SOCS-1. Conceivably, the binding of SOCS-3 to GHR tyrosines 333/338 could inhibit JAK2 signaling to STAT5 by interfering with JAK2 binding to the adjacent GHR box 1 region (residues 298–311), thereby raising the K_d of the receptor-kinase complex. Alternatively, SOCS-3 may directly bind to, and thereby inhibit, JAK2 via SOCS-3 sequences that are homologous to the kinase inhibitory region of SOCS-1 (24). In this model, SOCS-3 would serve as an inhibitory "bridge" by binding simultaneously to GHR phosphotyrosines 333/338 via its SH2 domain (SOCS-3 residues 46–141) and to JAK2 via its kinase inhibitory sequence (SOCS-3 residues 22–34). This latter sequence is conserved (8/13 similarity) with the corresponding region of SOCS-1 (residues 56–68) (24). The weak association of SOCS-3 with JAK2 than can be seen in the absence of receptor (27) is consistent with this proposal. Interestingly, the residues corresponding to the SOCS-1 kinase inhibitory sequence are poorly conserved in CIS and are altogether absent from SOCS-2 (data not shown). The "bridge model" proposed here for SOCS-3 inhibition of GH signaling may be more generally applicable to other cytokine signaling pathways, insofar as SOCS-3 inhibition of interleukin-2-activated JAK2 kinase is also markedly enhanced in the presence of receptor (47). Further study will be necessary to distinguish between these and other possible mechanisms for SOCS-3 inhibition.

The signaling inhibitory potential now demonstrated for four GH-inducible SOCS/CIS proteins strongly supports the proposed role of these proteins in negative regulation of GH signaling. GH signaling and transcriptional responses are distinct from those of many other hormones and cytokines by virtue of the sex-dependent temporal patterns of pituitary GH secretion, which confer the observed sexually dimorphic physiological responses to GH, such as pubertal growth rates (51) and liver P450 gene expression (52–54). STAT5b is a key mediator of the *in vivo* responses of rodents to the pulsatile plasma GH profile that is characteristic of males (6, 9, 10, 55). Further elucidation of the role of SOCS/CIS proteins in GH signaling will provide for a fuller understanding of the multiple physiological responses to GH, including the repeated activation and deactivation of signaling to liver STAT5b that is stimulated by sequential male plasma GH pulses (6) and the low level STAT5b signaling that becomes established in response to the female plasma GH pattern (7, 11). Detailed studies of the sex dependence and temporal expression of SOCS/CIS proteins in liver and other GH-responsive tissues following GH pulse stimulation or continuous GH treatment should provide insight into the important question of which GH signaling step(s) are regulated by each GH-inducible SOCS/CIS protein. Potential steps for SOCS/CIS regulation include: 1) binding to either GHR or JAK2 to effect the initial down-regulation of receptor-kinase

activity that begins ~40 min into a GH pulse (12); 2) binding to the GHR-JAK2 complex during a GH interpulse interval, which could contribute to the decreased responsiveness of liver cells to a second hormone pulse that we have observed during this refractory time period (12, 36); and 3) binding to GHR or JAK2 in cells exposed to GH chronically, which could contribute to the down-regulation of the GHR-JAK2-STAT5b signal transduction pathway that characterizes adult female rat liver (11).

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Addendum—The inhibitory actions of CIS on liver GH signaling were recently confirmed *in vivo* by Matsumoto *et al.* (56) in transgenic mice that express the CIS gene in liver constitutively. These mice displayed impaired liver STAT5b signaling, a decrease in body growth rates beginning at puberty and a loss of the male-dominant major urinary protein MUP, all characteristic of STAT5b-deficient mice (9, 10). Other data presented in that report confirm the inhibitory effects of CIS on GH-activated STAT described in this study, and indicate that the inhibitory action of CIS can be reduced in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient CIS inhibition of STAT5b transcriptional activity is achieved at lower transfected STAT5b levels, supporting the hypothesis that STAT5b and CIS compete for binding sites on GHR.

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