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) 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 pulsatile 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-
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 NH2-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, SS-1; SOCS-2 = CIS-2, SS-2; SOCS-3 = CIS-3, SS-3; and SOCS-6 = CIS-4. Mouse STAT5a and STAT5b expression plasmids pME18S (33) were obtained from Dr. A. Mui (DNAX Corp., Palo Alto, CA). Rat GHR cloned into pcDNA1/Amp or into pLM108, a plasmid that contains a metallothionein promoter and an SV40 enhancer (34), and derivatives of pLML10 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 pPK5 was obtained from Dr. N. Ihle (St. Jude’s, Memphis, TN). The STAT5 reporter plasmid 4X-pT719-Luc, which contains four copies of a STAT5 response element from the rat npt 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 (337, 377, or 800 ng) at 12.5 μg/100 g body weight by intraperitoneal injection. Longer term GH treatment was by hormone infusion at 2 μg/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—COS-7 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-pT719-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) for 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 activities 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, electrophoresized 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 408–438; 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 extracts, as described previously (37). Membranes 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 X-ray film.

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 OD600 nm of 0.9, treated with isopropyl-1-thio-galactoside (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 benzamidase, 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 immuno-precipitated 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 in vivo GH treatment, hypophysectomized rats were treated with a pulse of GH, followed by Northern blot analysis of CIS.
mRNA levels. Fig. 1A 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. 1A, lanes 7 and 8). In experiments carried out using the GH-responsive rat liver cell line CWSV-1 (36), GH given 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 A, 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.

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 either STAT5b (panels C and D) or STAT5a (panel A) 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 for cells transfected with STAT5a in the presence of CIS or SOCS/CIS proteins. The effects of the indicated SOCS/CIS proteins on STAT5b-stimulated ntcp reporter activity, determined using a dual luciferase assay method. Normalized luciferase activity is graphed as fold induction values relative to ntcp reporter activity without GH stimulation (mean ± S.D., n ≥ 4).
SOCS/CIS proteins. Migrations of protein size markers were as indi-
reprobed with anti-myc antibody to visualize each of the transfected
using the resolved on the blot shown were previously identified as unphosphoryl-
inhibited with GHR, STAT5b, and the indicated SOCS/CIS proteins and
the effects of these SOCS proteins on STAT5b EMSA activity. STAT5b EMSA inhibition (e.g. Fig. 2), phosphor-
yphosphorylated STAT5b could readily be detected in cells transfected with a low level of exogenous JAK2 (25 ng of plasmid) (Fig. 4A, lane 8). JAK2 phosphorylation in these cells was constitutive (i.e. GH-indepen-
dependent) (lane 7 versus lane 8). Interestingly, this constitutive
phosphorylated JAK2 is present at a level that is just barely detectable when analyzed by immunoprecipitation followed by anti-phosphotyrosine Western blotting (Fig. 4A, lane 2 versus lane 1). By contrast, tyrosine-phosphorylated JAK2 could
activate and thus may proceed via an indirect mechanism.
activity, which was assayed using a highly responsive
the GHR/JAK2 complex in a manner that interferes with the observed inhibitions may result from SOCS/CIS binding to
activation. These two possibilities were distinguished in exper-
mechanism. Thus, in contrast to SOCS-1, the inhibition of JAK2 phosphorylation by
exogenous JAK2 (25 ng of plasmid) (Fig. 4, lanes 1 and 2 versus lanes 3 and 4; cf. Fig. 2A, lanes 1 and 2).
transfected with GHR, STAT5b, and the indicated SOCS/CIS proteins and
treatment with GH as described in Fig. 2A. The major STAT5b bands
resolution on the blot shown were previously identified as unphosphoryl-
specific effects that GH has on the liver (10).
actially GH-independent activation of STAT5b (Fig. 4C, lanes 1 and 2 versus lanes 3 and 4; cf. Fig. 2A, lanes 1 and 2).
Western blot with antibody to the myc tag confirmed the ex-
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 lig-
activity, which in turn leads to JAK2 kinase activation and trans-phosphorylation. As expected, the constitu-
the importance of JAK2 kinase in GH/STAT5 signal transduction 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 di-
absence of GHR and thus may proceed via an indirect mechanism. Two potential mecha-
respect to its DNA binding specificity (40, 41) and its role in the sex-dependent and gene-
Western blot of COS-1 cells transfected with GHR, STAT5b, and the indicated SOCS/CIS proteins and
treatment with GH as described in Fig. 2A. 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.
STAT5b tyrosine phosphorylation and DNA binding (Fig. 4C; 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. 4D, lanes 9 and 10 versus lanes 1 and 2), while the other SOCS/CIS proteins exhibited much less or no inhibitory activity (Fig. 4D, lanes 3–8; cf. Fig. 4B). 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-
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 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. 5B 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).

**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 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).

**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. 6D, 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 numbering system, where residue 620 is the COOH-terminal amino acid, in contrast to rat GHR numbering system, where residue 620 is the COOH-terminal amino acid.

SOCS/CIS Inhibition of STAT5 Signaling

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. 5B 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. Re-probing of the Western blot with antibody to GST confirmed the recovery of GST fusion protein in the SOCS-3 samples (panel C). In addition, re-probing 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. 3A.

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. 6A, 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. 6C, lanes 5 and 6, and data not shown). SOCS-6 did not bind to any of the GHR fusion proteins (Fig. 6C, 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). Repробing 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. 6A), 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. 7A, 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. 7A, 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. 7A, bottom). Together, these findings demonstrate a requirement for tyrosines 333 and/or 338 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. 7B). 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. 7A). 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. 7C, SOCS-3 was inhibitory to STAT5 activation supported by GHRs with single tyrosine to phenylalanine mutations, GHR-Y333F and GHR-Y338F. Interestingly, partial
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 proteosomal 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 GH-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. 4D). 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 NH2-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 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 ≥ 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.

**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
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. 7C) 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 GH-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 GH. 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 inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient inhibition of CIS can be achieved in cells transfected with high levels of STAT5b. We have recently confirmed that much more efficient