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Role of the Cytokine-inducible SH2 Protein CIS in Desensitization of STAT5b Signaling by Continuous Growth Hormone*

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Growth hormone (GH)-inducible suppressors of cytokine signaling (SOCS/CIS proteins) inhibit GH receptor (GHR) signaling to STAT5b via phosphotyrosine-dependent binding interactions with the tyrosine kinase JAK2 (SOCS-1) and/or the cytoplasmic tail of GHR (CIS and SOCS-3). Presently, we investigate the mechanism of CIS inhibition and CIS's role in down-regulating GHR-JAK2 signaling to STAT5b in cells exposed to GH continuously. CIS is shown to inhibit GHR-JAK2 signaling by two distinct mechanisms: by a partial inhibition that is decreased at elevated STAT5b levels and may involve competition between CIS and STAT5b for common GHR cytoplasmic tail phosphotyrosine-binding sites; and by a time-dependent inhibition, not seen with SOCS-1 or SOCS-3, that involves proteasome action. Investigation of the latter mechanism revealed that GH stimulates degradation of CIS, but not SOCS-3. The proteasome inhibitor MG132 blocked this protein degradation and also blocked the inhibitory action of CIS, but not that of SOCS-1 or SOCS-3, on STAT5b signaling. Proteasome-dependent degradation of CIS, most likely in the form of a (GHR-JAK2)-CIS complex, is therefore proposed to be an important step in the time-dependent CIS inhibition mechanism. Finally, the down-regulation of GHR-JAK2 signaling to STAT5b seen in continuous GH-treated cells could be prevented by treatment of cells with the proteasome inhibitor MG132 or by expression of CIS-R107K, a selective, dominant-negative inhibitor of CIS activity. These findings lead us to propose that the cytokine signaling inhibitor CIS is a key mediator of the STAT5b desensitization response seen in cells and tissues exposed to GH chronically, such as adult female rat liver.

Cell signaling via the growth hormone (GH)[1] receptor (GHR) is characterized by the activation of multiple intracellular transduction pathways, including those leading to the activation of STATs, MAP kinase, phosphatidylinositol 3′-kinase, and protein kinase C (1, 2). STAT activation occurs via a GH-inducible tyrosine phosphorylation reaction catalyzed by the GHR-associated tyrosine kinase JAK2. In liver, an important GH target tissue, two closely related STAT proteins, STAT5a and STAT5b (>90% identical), bind to the tyrosine-phosphorylated GHR, enabling the STAT to undergo rapid tyrosine phosphorylation *in vivo* in response to physiological levels of circulating GH (3). This, in turn, leads to dimerization and nuclear translocation of the activated STAT, which can directly bind to target gene DNA response elements and stimulate gene transcription (4). In contrast, GH activation of STATs 1 and 3 requires high GH concentrations to proceed efficiently (5) and appears to involve direct interactions between JAK2 and the STAT, without a need for the specific GHR cytoplasmic domain phosphotyrosine residues that serve as docking sites for activation of STAT5 (6–8).

Many physiological responses to GH are critically dependent on the temporal pattern of plasma GH stimulation (9). This dependence is most striking in rodent liver, where the intermittent occurrence of strong, well defined plasma GH pulses that is characteristic of adult males stimulates transcription of a set of cytochrome P450 and other genes that is distinct from the set of genes that is transcriptionally activated by the more continuous plasma GH profile associated with adult females (3, 10). The sexual dimorphism of GH-regulated liver gene expression is critically dependent on STAT5b, as revealed by mouse knockout studies (11–14), supporting the proposal that liver STAT5 (primarily STAT5b) is a key mediator of the effects of plasma GH pulses on the male pattern of liver gene expression (15). Liver STAT5b is strongly, and repeatedly, activated in adult male rats in response to each incoming plasma GH pulse (16), while in adult female rats the near continuous presence of GH in plasma leads to substantial down-regulation of GHR-JAK2-dependent signaling to STAT5b (17). This down-regulation of signaling from GHR-JAK2 to STAT5b is readily evident in liver cells exposed to GH continuously, where the down-regulation requires protein synthesis (18, 19). Factors that regulate GHR internalization and degradation (20–23) may potentially be involved in the down-regulation of GHR-JAK2 signaling. Factors that modulate GH-stimulated STAT5 signaling, including cytoplasmic and nuclear phosphotyrosine phosphatases, such as SHP-1 and SHP-2 (24–26), protein inhibitors of activated STATs (27) and SOCS/CIS proteins (28, 29), may also contribute to the down-regulation of cellular GH responses.

SOCS/CIS proteins have been identified as potentially important feedback inhibitors of cytokine receptor signaling in a broad range of tissues and cell types. SOCS/CIS genes can be rapidly but transiently induced by a variety of cytokines and growth factors, including GH (30–34) and prolactin (35), via STAT-dependent transcriptional mechanisms (36, 37). Previous studies have demonstrated that SOCS/CIS proteins can inhibit GH signaling by multiple mechanisms. These include direct JAK2 inhibition, in the case of SOCS1; JAK2 inhibition

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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*, STAT-activated sodium-dependent taurocholate cotransporter gene; SH2, Src homology 2; GST, glutathione *S*-transferase; GFP, green fluorescence protein.

in a manner that is dependent on membrane proximal GHR phosphotyrosine residues, in the case of SOCS-3; and inhibition of GHR-JAK2 signaling via membrane-distal GHR phosphotyrosine residues, in the case of CIS and SOCS-2 (32, 38).

Although GH can induce multiple SOCS/CIS mRNAs in liver and other tissues, little is known about the physiological functions of the individual SOCS/CIS proteins, including their potential role in termination of GH pulse signaling and in mediating the down-regulation of GHR-JAK2 signaling to STAT5b that occurs *in vivo* in tissues chronically exposed to GH, such as adult female rat liver (15, 17). Recent studies reveal that SOCS-2-deficient mice exhibit gigantism and other phenotypes indicative of impaired negative regulation of the GH/insulin-like growth factor-1 axis (39). Analysis of *SOCS-1* and *SOCS-3* gene knock-out mice, which exhibit complex neonatal or embryonic defects (40–42), have not been informative in this regard. To further address the role of SOCS/CIS proteins in GH regulation, we presently investigate the GHR-dependent mechanisms through which CIS inhibits signaling to STAT5b. Our findings lead us to propose two distinct, but linked mechanisms for CIS inhibition: 1) direct competition with STAT5b for common phosphotyrosine-binding sites on the GHR cytoplasmic tail, followed by 2) proteasome-dependent degradation of CIS protein bound to the GHR-JAK2 signaling complex. We also characterize a CIS SH2 domain mutant, CIS-R107K, and extend earlier studies of its action in interleukin-2 signaling (43) to demonstrate its selective, dominant-negative activity toward CIS-inhibited GHR-JAK2 signaling. Finally, we use this CIS mutant in experiments that demonstrate a critical role for an endogenous, GH-inducible CIS protein in the down-regulation of STAT5b signaling that occurs in liver cells stimulated with GH continuously.

MATERIALS AND METHODS

Plasmids—pME18S expression plasmid encoding mouse STAT5b was obtained from Dr. Alice Mui (DNAX Corp., Palo Alto, CA). Rat GHR cloned into expression plasmid pLM108 was provided by Dr. Nils Billestrup (Hagedorn Research Institute, Gentofte, Denmark) and pFlag-CMV1-rabbit GHR expression plasmid was provided by Dr. Joelle Finidori (INSERM Unit 344, Paris Cedex, France). pCDNA3 expression plasmids encoding human CIS, SOCS-1, SOCS-2, and SOCS-3, each with an NH_2-terminal c-Myc tag (Dr. Akihiko Yoshimura, Kurume University, Kurume, Japan) (44) and STAT5 reporter plasmid 4x-pT109-Luc, which contains four copies of a STAT5 response element from the rat *ntcp* gene (Dr. Mary Vore, University of Kentucky, Lexington, KY) (45) were obtained from the sources indicated. Flag-tagged wild-type CIS expression plasmid cloned in pME18S, the arginine to lysine point mutation at CIS residue 107 (CIS-R107K) (43), and expression plasmids for the analogous SOCS-1-R105E and SOCS-1-R105K mutants were obtained from Dr. Warren Leonard, National Institutes of Health, Bethesda, MD.

COS-1 Cell Culture and Transfection—COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfection of COS-1 cells grown in 6-well tissue culture plates was carried out using FuGene 6 transfection reagent (Roche Molecular Biochemicals). GHR used in the transfection studies was rat GHR, unless indicated otherwise. Thirty h after addition of the DNA/FuGene 6 mixture to the cells (~1.1–1.2 μl of FuGene 6/1 μg of total DNA), cells were stimulated with rat GH (National Hormone and Pituitary Program, NIDDK; rGH-B-14-SIAFP) at concentrations specified in the figure legends. Cells were lysed at different times, whole cell extracts were prepared using lysis buffer (Tropix, Inc.) and firefly luciferase and *Renilla* luciferase activities were measured 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 based on $n = 2$ or 3 replicates and are representative of at least two or three independent sets of experiments.

EMSA and Western Blot Analysis—EMSA analyses using probe for STAT5 DNA binding activity was performed as described previously (46). For Western blotting, whole cell lysates from transfected COS-1 cells were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Western blotting of SOCS/CIS proteins was performed using monoclonal antibody raised to a Myc epitope tag (c-Myc amino acids 408–439, Santa Cruz Biotechnology sc-40; 1/1000 dilution) (32) or a rabbit polyclonal antibody to a Flag epitope tag (Santa Cruz sc-807; 1/1000 dilution), as indicated. STAT5b Western blotting used anti-STAT5b polyclonal antibody sc-835 from Santa Cruz Biotechnology as described elsewhere (32).

Confocal Microscopy and Immunofluorescence Analysis—CWSV-1 cells grown in serum-free RPCD medium (46) were seeded onto 22-mm glass coverslips in 35-mm Corning dishes containing RPCD medium with 3% fetal bovine serum (25). Cells isolated from one confluent 100-mm dish were used to seed 15–18 coverslips at a volume of 0.25–0.3 ml/coverslip. Cells were allowed to adhere for 2 h, and then were incubated for 10 h in RPCD medium without serum. Cells were then transfected using FuGene 6 with expression plasmid encoding GFP (pEGF2-N2; CLONTECH; 250 ng), either alone or in combination with CIS (500 ng), CIS-R107K (2 μg), or SOCS-3 (250 ng). Twenty-four h after addition of the DNA/FuGene 6 mixture to the cells, the cells were stimulated with GH (500 ng/ml) for the indicated times. Cells were then fixed with methanol for 20 min at −20 °C, blocked twice with 3% calf serum in phosphate-buffered saline for 10 min, and then incubated with rabbit polyclonal anti-STAT5b antibody (0.2 μg/ml) as described (25). Following two washes with phosphate-buffered saline containing 3% calf serum, the coverslip was incubated while inverted on a parafilm strip, with 0.04 ml of secondary antibody (1:500 dilution in phosphate-buffered saline containing 3% calf serum) in the dark for 1 h at 37 °C. The samples were washed twice in 3% calf serum and once with phosphate-buffered saline and then mounted onto glass slides. Fluorescence was visualized using an Olympus BX-50 Confocal LaserScanning Microscope. Primary antibody was detected using Alexa Fluor 594-conjugated goat anti-rabbit IgG second antibody (Molecular Probes, Inc., Eugene, OR) (excitation at 568 nm and detection through a 610-nm long pass filter (red channel)). Transfected cells were identified by the green fluorescence of the co-transfected GFP expression plasmid (excitation using 488 nm krypton-argon laser, with emission detected through a 530-nm band pass filter (green channel)). Image files were processed for presentation using Adobe Photoshop.

RESULTS

CIS Inhibits STAT5b Signaling More Completely at Lower STAT5b Levels—Previous studies have shown CIS and SOCS-2 are partially inhibitory to GH-stimulated STAT5b activation in transfected COS-1 cells under conditions where SOCS-1 and SOCS-3 are fully inhibitory (32). To investigate the mechanism for the partial inhibition by CIS, COS-1 cells were transfected with GHR and CIS expression plasmids in the presence of varying amounts of STAT5b expression plasmid. GH-stimulated STAT5b activation was assayed by EMSA analysis of STAT5b DNA binding activity and by measuring luciferase activity using a STAT5 response element-luciferase reporter derived from the rat *ntcp* gene. Cells were treated with GH for 5 h, to allow for expression of the luciferase reporter. As shown in Fig. 1, partial inhibition of STAT5b EMSA activity (*panel A*) and ntcp-luciferase reporter activity (*panel B*) was seen in cells transfected with 500 ng of STAT5b expression plasmid, equivalent to the amount of STAT5b plasmid transfected in our earlier studies (32). In cells transfected with lower amounts of STAT5b expression plasmid, resulting in correspondingly lower levels of STAT5b activity after GH stimulation (*panel A, top, lanes 2–9*), CIS exerted a more complete inhibition of STAT5b EMSA activity (*panel A*) and luciferase reporter activity (*panel B*). A similar STAT5b dependence of CIS inhibitory activity was seen when STAT5b EMSA activity was monitored in cells treated with GH for 30 min (data not shown). Given that STAT5b (47, 48) and CIS (32, 38) apparently bind to an overlapping set of GHR cytoplasmic tail phosphotyrosine residues, this finding suggests that the partial nature of the inhibition at higher STAT5b levels reflects competition between CIS and STAT5b for common GHR-binding site(s). In contrast, the inhibitory effect of SOCS-3 was essentially complete over a broad STAT5b range (Fig. 1). This is consistent with our finding that SOCS-3 preferentially binds to the membrane-proxi-

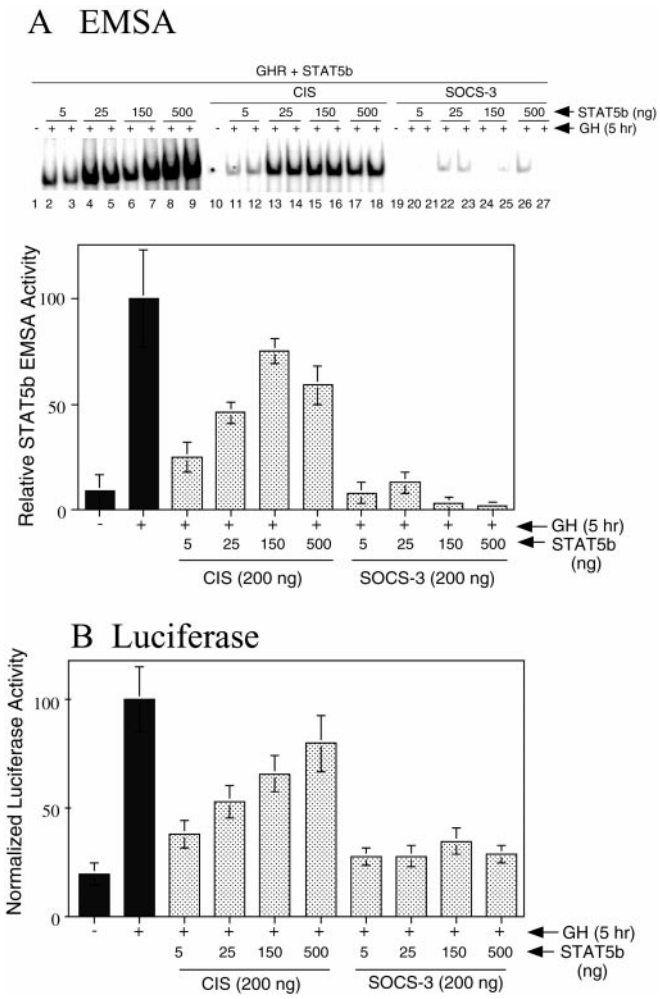


FIG. 1. **CIS inhibition of STAT5b signaling is more complete at lower STAT5b levels.** COS-1 cells were transfected with expression plasmids encoding rat GHR (100 ng), reporter plasmid 4x-pT109-Luc (400 ng), CIS (200 ng), or SOCS-3 (200 ng) together with increasing amounts of STAT5b (5, 25, 150, or 500 ng). A *Renilla* luciferase expression plasmid (pRL-TK) (40 ng) was included to normalize samples for transfection efficiencies. After transfection, cells were stimulated for 5 h with 100 ng/ml rat GH. Cell lysates were then prepared and analyzed for STAT5 DNA binding activity by EMSA using a β-casein DNA probe (*panel A*) or assayed for normalized luciferase reporter activity (*panel B*). STAT5b protein levels detected by Western blot were maximal at the 150-ng plasmid level (data not shown). *Panel A,* EMSA gel of STAT5 DNA binding activity, assayed using β-casein probe, in extracts of cells transfected with the indicated factors and then stimulated with GH for 5 h. CIS inhibits GH-inducible STAT5b transcriptional activity more completely at low STAT5b levels, whereas the inhibition by SOCS-3 is already complete at high STAT5b levels. STAT5b EMSA activity in the presence of CIS or SOCS-3 is graphed at each STAT5b plasmid level relative to the corresponding GH stimulated activity obtained in the absence of SOCS/CIS expression plasmid (*i.e.* STAT5 activity for samples in *lanes 2–9,* respectively serve as the 100% values for the samples in *lanes 11–18* and *20–27*). *Panel B,* firefly luciferase reporter activity normalized for the level of *Renilla* luciferase activity is graphed as a percentage of the GH stimulated activity obtained in the absence of CIS or SOCS-3 expression (mean ± S.D., $n = 4$).

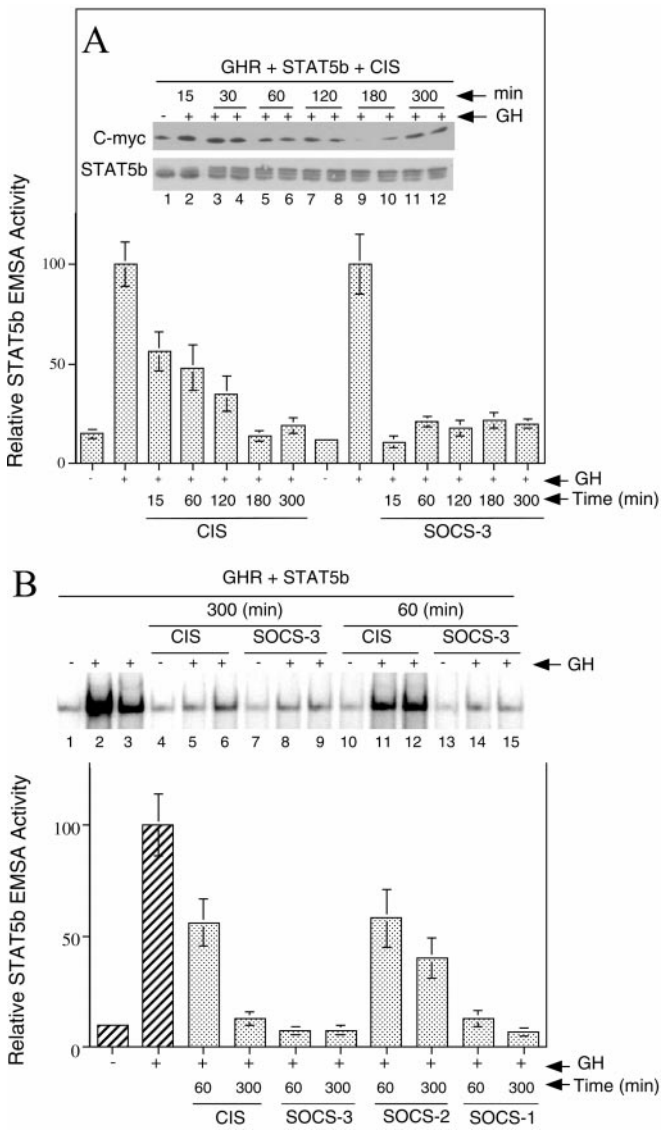


FIG. 2. **Time course of SOCS/CIS protein inhibition of STAT5b EMSA activity.** COS-1 cells were transfected with expression plasmids encoding rabbit GHR (10 ng), STAT5b (25 ng), and each of the indicated SOCS/CIS proteins: CIS (200 ng), SOCS-1 (200 ng), SOCS-2 (500 ng), or SOCS-3 (200 ng). Thirty h later, cells were treated with 200 ng/ml GH for times ranging from 15 to 300 min, followed by preparation of whole cell extracts used for EMSA assays of STAT5 DNA binding activity. *Panel A,* EMSA analysis showing that CIS protein exerts a complete inhibition of STAT5b DNA binding activity only after 3 h, whereas the inhibitory effect of SOCS-3 is already maximal at 15 min. *Inset,* Western blot analysis of CIS protein (detection of c-Myc tag) and STAT5b protein (multiple bands correspond to variously phosphorylated STAT5b forms (46)) in duplicate samples co-transfected with GHR, STAT5b, and CIS expression plasmids. *Panel B,* EMSA gel and quantitative EMSA data showing GH-inducible STAT5b DNA binding activity is fully blocked by SOCS-3 and SOCS-1 after 60 min and by CIS only after 300 min. Activity is partially blocked in cells expresing SOCS-2 at both 60 and 300 min.

mal GHR phosphotyrosine residues 333 and/or 338 (32), which are distinct from the more COOH-terminal major GHR-binding sites for STAT5b identified earlier (47, 48).

CIS Inhibitory Effect Is Time-dependent—CIS inhibition of STAT5b-stimulated ntcp-luciferase reporter activity, measured after 24 h GH treatment, is more complete than the inhibition of STAT5b EMSA activity, measured after 30 min GH treatment (32). To ascertain whether this discrepancy reflects a time dependence of the inhibitory action of CIS, COS-1 cells transfected with GHR, STAT5b, and CIS were stimulated with GH for times ranging from 15 to 300 min, at which time cell extracts were assayed for STAT5b EMSA activity. As seen in Fig. 2*A*, the inhibitory effect of CIS on STAT5b EMSA activity was increased as a function of time after GH treatment, becoming maximal by 180 min. Western blotting confirmed the consistency of cellular STAT5b protein levels during the course of GH treatment (Fig. 2*A, inset*). In contrast to CIS, SOCS-1 and SOCS-3 were already maximally inhibitory at 15–60 min, while SOCS-2 was partially inhibitory, even after 300 min GH

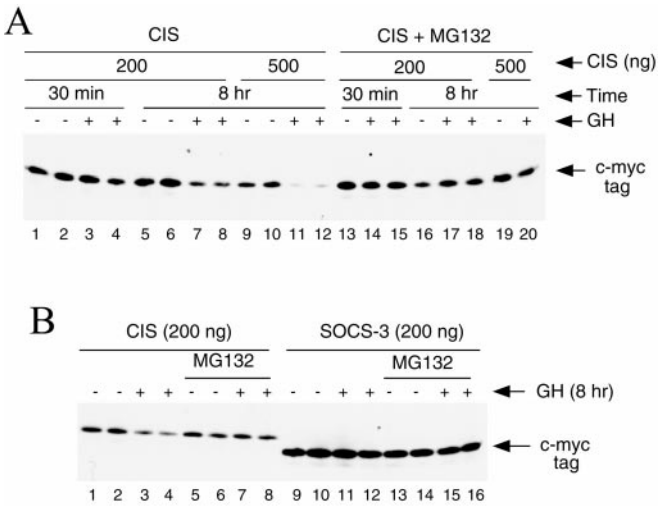


FIG. 3. **Proteasome inhibitor MG132 prevents GH-induced decrease in CIS protein.** Shown in *panels A* and *B* are c-Myc Western blots showing the time dependence of GH-stimulated CIS protein degradation and the protective effect of MG132 in transfected COS-1 cells. Cells were transfected for 30 h with expression plasmids encoding GHR (200 ng), STAT5b (50 ng), and c-Myc-tagged CIS or SOCS-3 (200–500 ng, as indicated). Cells were then treated with 50 μM MG132 for 15 min, as indicated, followed by GH stimulation (50 ng/ml) for 30 min to 8 h. Cell extracts were then prepared and blotted with anti-Myc antibody to visualize each of the transfected CIS or SOCS-3 proteins. *Panel A* shows substantial GH-induced loss of CIS protein in cells transfected with higher levels of CIS plasmid (*lanes 9–12 versus 5–8*) which is blocked by MG132 (*lanes 13–20 versus 1–12*). In the absence of MG132, CIS but not SOCS-3 protein is degraded in a GH-dependent manner (*panel B, lanes 3* and *4 versus 11* and *12*).

treatment (*panel B* and data not shown). The time dependence of the inhibitory action of CIS, but not that of the other SOCS/CIS proteins, further demonstrates that CIS inhibits signaling from the GHR·JAK2 complex to STAT5b in a manner that is fundamentally different from the other SOCS/CIS proteins.

GH-stimulated, Proteasome-dependent Degradation of CIS Protein—We next examined whether the time-dependent nature of CIS inhibition is associated with GH-stimulated CIS protein degradation. COS-1 cells transfected with GHR, STAT5b, and CIS were stimulated with GH, and cell extracts were then assayed for CIS protein, detected by Western blotting using an antibody directed at the cloned protein's NH_2-terminal c-Myc tag. Short-term GH treatment, associated with partial inhibition of STAT5b activity (Fig. 2), did not lead to a decrease in CIS protein levels (Fig. 3*A, lanes 3* and *4 versus 1* and *2*). By contrast, longer term GH treatment resulted in a significant decline in CIS protein (Fig. 3*A, lanes 7* and *8 versus 5* and *6*; also see *inset* of Fig. 2*A*). No such GH-stimulated decrease was seen in the case of SOCS-3 protein (Fig. 3*B, lanes 9–12 versus 1–4*). Treatment of the cells with the proteasome inhibitor MG132 (50 μM) (49), added 15 min prior to GH, effectively prevented the GH-induced degradation CIS (Fig. 3*A, lanes 13–20 versus 1–12*; Fig. 3*B, lanes 5–8 versus 1–4*). Thus, the proteasome pathway participates in GH-induced CIS degradation. During the course of these studies we observed that the loss of CIS protein was, paradoxically, more complete when the cells were transfected with higher amounts of CIS expression plasmid (Fig. 3*A*, *lanes 9–12 versus 5–8*). The reason for this latter effect is unclear. However, this effect was not seen in cells treated with MG132 (*lanes 16–20*), suggesting a role for the proteasome in this process.

Proteasome Inhibitor MG132 Selectively Blocks the Long-term Inhibitory Action of CIS—We next investigated whether the GH-stimulated, proteasome-mediated degradation of CIS shown in Fig. 3 contributes to the inhibitory effects of CIS on STAT5b signaling. COS-1 cells were transfected with GHR, STAT5b, and ntcp-luciferase reporter in the presence or absence of CIS. Cells were pretreated with MG132 (15 min) followed by GH addition. ntcp-Luciferase reporter activity was assayed 8 h later. Under these conditions, GH stimulated a 7.5-fold increase in STAT5b-stimulated reporter gene activity (Fig. 4*A, first two bars*). This increase was blocked by ~90% in CIS-transfected cells, as expected from the extensive inhibition of STAT5b EMSA activity by the 5-h time point shown in Fig. 2. Moreover, CIS inhibition of reporter activity was substantially prevented by MG132 treatment (Fig. 4*A, dark bars*). This indicates that the MG132-sensitive, GH-dependent degradation of CIS protein seen in the same experiments (Fig. 3) is mechanistically linked to the time-dependent inhibitory action of CIS on STAT5b signaling. Western blot analysis of STAT5b protein levels revealed that the protective effect of MG132 on STAT5b EMSA activity is associated with an increase in relative abundance of the active, tyrosine-phosphorylated form of STAT5b (Fig. 4*B*, uppermost STAT5b band; *lanes 11* and *12 versus lanes 7* and *8*). No such effect of MG132 was seen in the case of SOCS-3 (data not shown).

Further study revealed that the protective effect of MG132 was only observed at longer times of GH treatment. Thus, the partial inhibitory action of CIS on STAT5b EMSA activity seen after a 30-min period of GH stimulation was not blocked by MG132 (Fig. 4*C, top, lanes 10* and *11 versus lanes 4* and *5*, as compared with *lanes 12* and *13 versus lanes 6* and *7*; also see quantitation in Fig. 4*C, bottom*). Finally, in control experiments, the protective effect of MG132 was shown to be specific to the inhibitory action of CIS, as a corresponding protection was not seen with three other inhibitory SOCS/CIS proteins (Fig. 4*A*).

Characterization of Dominant-negative CIS SH2 Domain Mutant R107K—CIS-R107K contains a point mutation within CIS's SH2 domain (Arg^{107} to Lys) that enhances interleukin 2-stimulated STAT5 transcriptional activity in transfected cells and may act in a dominant-negative fashion to block an endogenous CIS activity (43). Characterization of CIS-R107K with respect to GH signaling revealed that this SH2 domain mutant does not exhibit the strong time-dependent inhibition of signaling to STAT5b seen with wild-type CIS, both when assayed at the level of STAT5b EMSA activity (Fig. 5*A, top, lanes 5* and *6 versus lanes 2* and *3*; also see *bar 3 versus bar 4*) and at the level of ntcp-luciferase reporter activity (Fig. 5*B*). Moreover, co-transfection of CIS-R107K with wild-type CIS substantially reversed the inhibitory action of the wild-type inhibitor on STAT5b EMSA activity and on luciferase reporter activity (Fig. 5, *A* and *B, bar 5 versus bar 4*), thereby demonstrating the dominant-negative activity of CIS-R107K. Western blot analyses verified that co-transfection of CIS-R107K (Flag-tagged) with CIS did not decrease expression of CIS protein (Myc-tagged) (Fig. 5*C, lanes 9–16 versus lanes 1–4*) or STAT5b (data not shown). The dominant-negative activity of CIS-R107K was specific to CIS, as it was not manifest when GH signaling was inhibited by SOCS-1, SOCS-2, or SOCS-3 (Fig. 5, *A* and *B, last 6 bars*).

To gain insight into the mechanism that governs the dominant-negative action of CIS-R107K, we investigated the intrinsic capacity of the CIS mutant to bind the GHR cytoplasmic tail. *Escherichia coli*-expressed glutathione *S*-transferase (GST)-GHR fusion proteins comprising COOH-terminal residues 291–620 or 540–620 of GHR (8) were tyrosine phosphorylated by a co-expressed *Elk* tyrosine kinase and then incubated with extracts of COS-1 cells transfected with CIS, CIS-R107K, or CIS in combination with CIS-R107K. GST-GHR-bound proteins isolated on glutathione-agarose beads were

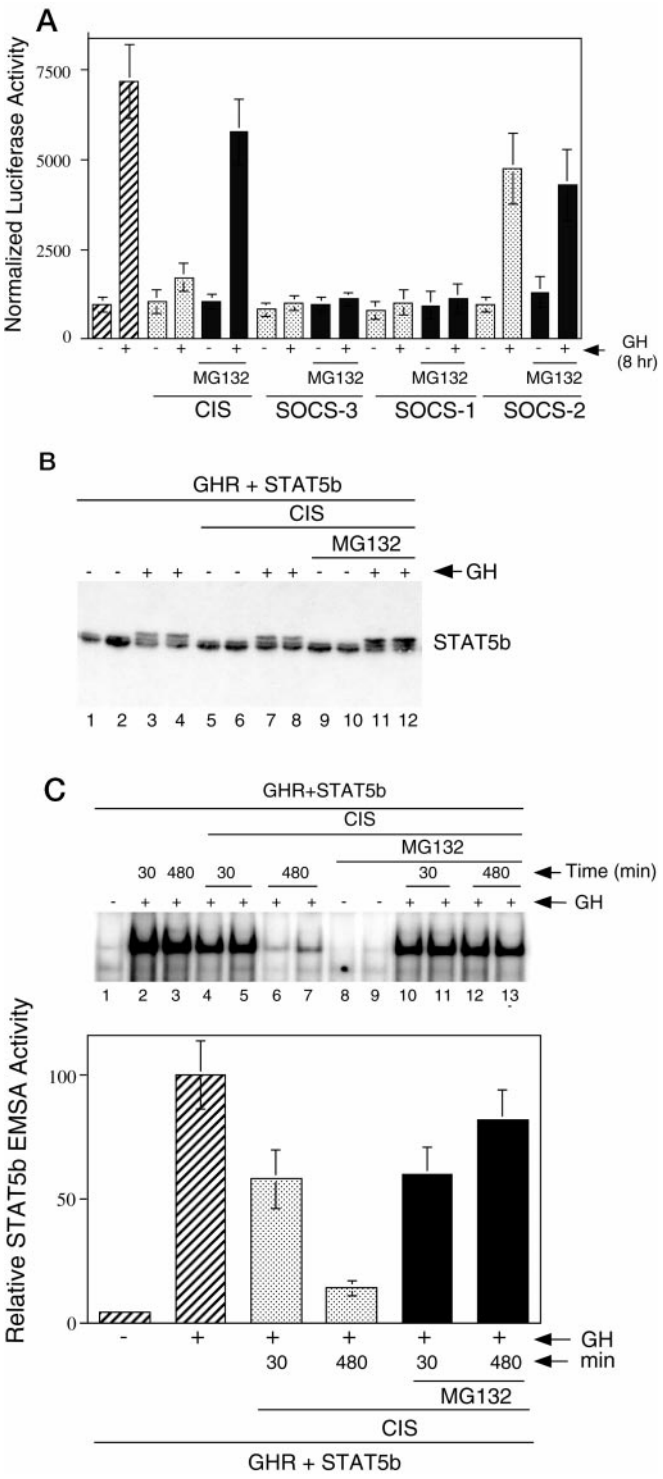


FIG. 4. **Proteasome inhibitor MG132 blocks CIS inhibition of GH-stimulated STAT5b activation.** *Panel A,* COS-1 cells transfected with expression plasmids encoding GHR (200 ng), STAT5b (50 ng), and the reporter gene 4x-pT109-Luc were co-transfected with CIS (200 ng), SOCS-1 (50 ng), SOCS-2 (500 ng), or SOCS-3 (200 ng), as indicated. Cells were stimulated with GH (50 ng/ml) for 8 h, and normalized luciferase reporter activity was assayed. MG132 is shown to block the inhibitory effects of CIS but not that of SOCS-1, SOCS-2, or SOCS-3. The CIS and SOCS-3 Western blot of these samples is shown in Fig. 3*B*. *Panel B,* Western blot of STAT5b in samples corresponding to control and CIS-transfected groups shown in *panel A*. Uppermost band seen in *lanes 3, 4, 7, 8, 11*, and *12* corresponds to the tyrosine-phosphorylated form of STAT5b (46). This band is decreased in relative abundance in CIS-transfected samples (*lanes 7* and *8 versus lanes 3* and *4*) but is maintained at the uninhibited control levels in the presence of MG132 (*lanes 11* and *12*). *Panel C,* experiment carried out as described under *panel A*, except that the cells were stimulated with GH for either 30 or 480 min, as indicated, and STAT5 EMSA activity was assayed. Shown is a representative EMSA gel along with quantitative data based on two experiments. The CIS and SOCS-3 Western blot corresponding to *panel B* is shown in Fig. 3*A*.

visualized on Western blots probed with anti-Myc antibody (detection of wild-type CIS; Fig. 5*D, upper panel*) or anti-Flag antibody (detection of CIS-R107K; Fig. 5*D, middle panel*). Recovery of the GST-GHR fusion protein was verified by reprobing with antibody to GST (Fig. 5*D, lower panel*). In agreement with our earlier findings (32), CIS bound well to both the short and long GHR fragments, but only in their tyrosine-phosphorylated forms (Fig. 5*D, upper panel, lanes 1, 3, 7,* and *9 versus lanes 2* and *8,* and data not shown). In contrast, CIS-R107K neither bound to the GHR fusion proteins (Fig. 5*D, lanes 4–6, middle panel*) nor inhibited the binding of wild-type CIS (compare band intensities for *top panel, lanes 7 versus 1* and *lanes 9 versus 3*), demonstrating a requirement for CIS's intact SH2 domain for GHR binding and for inhibition of signaling to STAT5b. Moreover, the absence of GHR binding by CIS-R107K indicates that the dominant-negative activity of this mutant is manifest at a step downstream of the GHR-CIS binding step. Indeed, CIS-R107K was not able to block the short-term inhibitory action wild-type CIS on STAT5b signaling, *i.e.* that seen 30 min after GH treatment (Fig. 5*E, bar 5 versus bar 4*). This latter finding provides further support for the conclusion that the short-term inhibitory actions of CIS are mechanistically distinct from those seen after longer term GH treatment.

CIS-R107K Blocks Continuous GH-induced Down-regulation of Signaling to STAT5b—The GH-stimulated, time-dependent inhibitory action of CIS described above suggests that the GH-inducible *CIS* gene (31, 32, 34) may play an important role in the time-dependent down-regulation of GHR-JAK2 signaling to STAT5b seen in liver cells exposed to GH continuously (15, 18). To test this hypothesis, we utilized the dominant-negative properties of CIS-R107K to probe for the role of endogenous CIS protein in the down-regulation of STAT5b signaling. Fig. 6 presents the time course for activation and deactivation of STAT5b EMSA activity in transfected COS-1 cells (Fig. 6, *A, lanes 1–7, B, first set of bars*). The progress of the activation phase (maximal by 60 min) and the down-regulation, or desensitization phase of STAT5b signaling (still incomplete at 300 min in these cells) is similar to, albeit slower than that seen in the liver cell line CWSV-1 (18). In cells co-transfected with CIS-R107K, however, no decline in STAT5b activity was apparent, even after 300 min of GH treatment (Fig. 6, *A, lanes 9–14, B, middle set of bars*). A similar result was obtained in cells treated with the proteasome inhibitor MG132 (Fig. 6, *A, lanes 15–20, B, last set of bars*), which we previously demonstrated blocks the down-regulation of STAT5b signaling by prolonging signaling from the GHR-JAK2 receptor-kinase complex to STAT5b (18, 50). Given the specificity of CIS-R107K for the inhibition of GHR signaling by CIS (Fig. 5, *A* and *B*), these findings suggest that the endogenous *CIS* gene, which is transcriptionally activated by STAT5 (36, 51), plays a key role in the down-regulation of signaling to STAT5b in cells exposed to GH continuously. Endogenous CIS expression in the GH-stimulated COS-1 cells was at a level too low to detect using anti-CIS antibody (Santa Cruz Biotechnology) (data not shown). This may in part explain the slow down-regulation of STAT5b signaling in COS-1 cells seen in Fig. 6.

The effect of CIS-R107K on the down-regulation of STAT5b signaling in transfected COS-1 cells was confirmed and extended in studies carried out in the liver cell line CWSV-1. Transient transfection in conjunction with a confocal fluorescence microscope-based single cell assay was used to evaluate

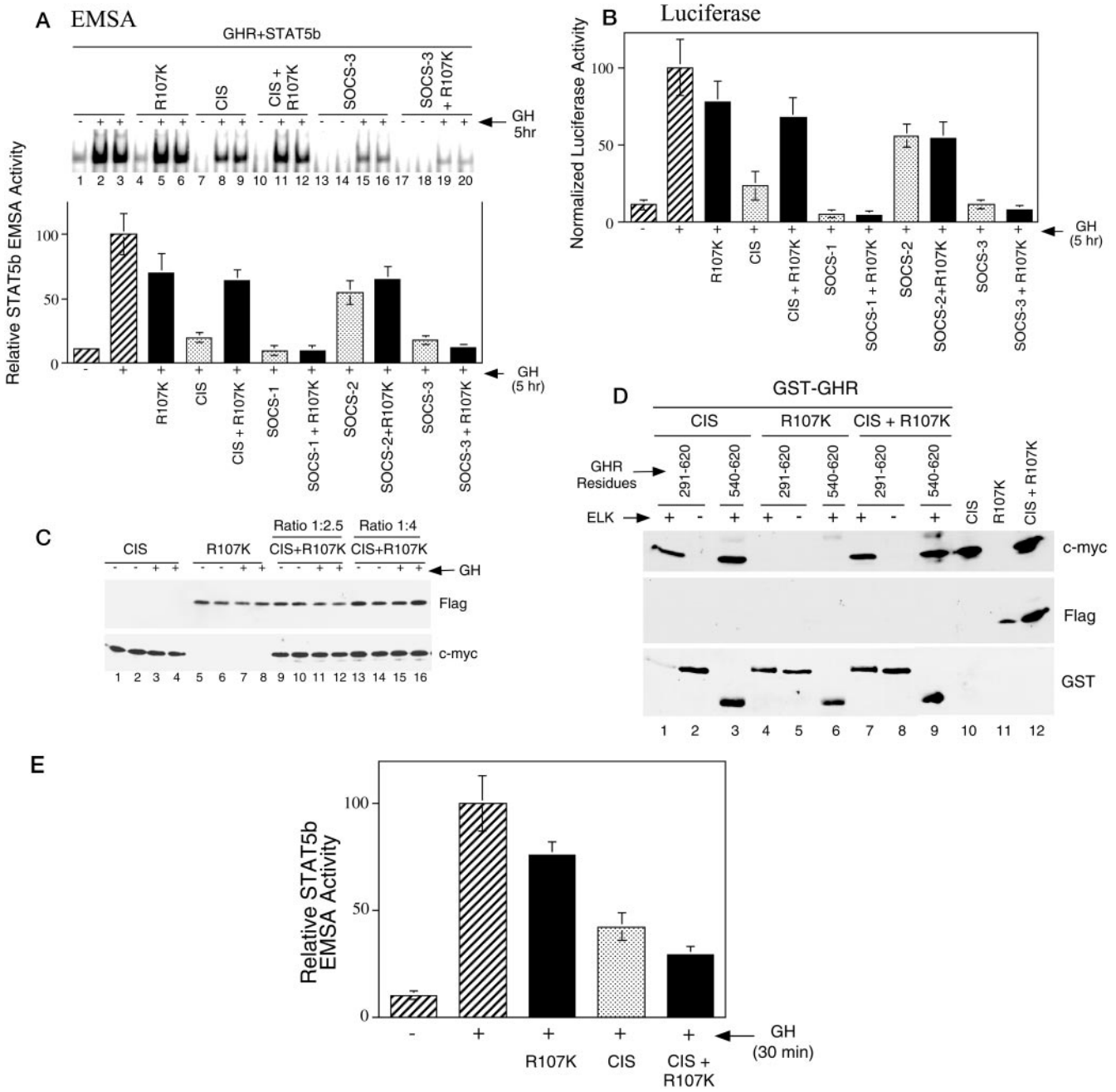


FIG. 5. **Dominant-negative CIS mutant R107K blocks inhibition of GH-inducible STAT5b activity by wild type CIS.** COS-1 cells were co-transfected with GHR (200 ng), STAT5b (100 ng), and the reporter plasmid 4x-pT109-Luc in the presence of CIS (200 ng), SOCS-1 (50 ng), SOCS-2 (500 ng), or SOCS-3 (100 ng) expression plasmids. Where indicated, CIS-R107K expression plasmid was co-transfected at 500 ng except in the case of SOCS-2, where 800 ng of CIS-R107K was used. Cells were stimulated with GH (200 ng/ml) for 5 h and relative EMSA (*panel A*) and luciferase reporter activities (*panel B*) were assayed. CIS-R107K blocks the inhibitory effect of wild-type CIS but not that of SOCS-1, SOCS-2, or SOCS-3. *Panel C,* Western blot of CIS (c-Myc epitope tag) and CIS-R107K protein (Flag tag) in transfections such as those shown in *panels A* and *B*. *Panel D,* bacterial extract (10 μl) containing equivalent amounts of GST-GHR fusion proteins (*c.f.* anti-GST probing, *lower panel*), which encompass amino acids 291–620 or 540–620 of the human GHR, were prepared in the presence or absence of the *Elk* tyrosine kinase, as indicated. To assay the binding of CIS or CIS-R107K to the GST-GHR fusion proteins, bacterial extracts bound to glutathione-agarose beads were incubated for 2.5 h at 4 °C with total extracts of COS-1 cells transfected with CIS (200 ng), CIS-R107K (500 ng), or CIS in combination with CIS-R107K (200 and 800 ng, respectively) (75 μg of COS-1 cell extract in 0.1 ml of fusion lysis buffer (32)). The beads were washed, eluted in SDS gel buffer, and then analyzed on a Western blot probed sequentially with anti-Myc, anti-Flag, and anti-GST antibodies, as indicated. Recovery of GST-GHR fusion protein was low in *lane 1. Lanes 10–12* contain 15 μg of the same SOCS/CIS-transfected COS-1 cell extracts used for the samples shown in *lanes 1–9*, but were loaded onto the SDS gel directly without binding to the GST-GHR fusion protein. Lower intensity of the Flag signal in *lane 11* compared with *lane 12* reflects the 4-fold increased level of transfected CIS-R107K plasmid in the latter sample. *Panel E,* experiment carried out as in *panels A* and *B* except that the cells were stimulated with GH for 30 min and STAT5 EMSA activity was assayed.

the effects of CIS-R107K on the STAT5b desensitization response in CWSV-1 cells exposed to GH continuously. Cells were transfected with CIS, CIS-R107K, or SOCS-3. Transfected cells were marked by co-transfection of the green fluorescence protein, GFP. STAT5b protein, which is endogenous to the cells, was visualized by indirect immunofluorescence using an Alexa Red-conjugated second antibody. Cells were stimulated with GH for 45 min, which leads to near-maximal STAT5b activation and nuclear translocation (25, 50), or were treated with GH continuously for 180 min, at which point GHR-JAK2 sig-

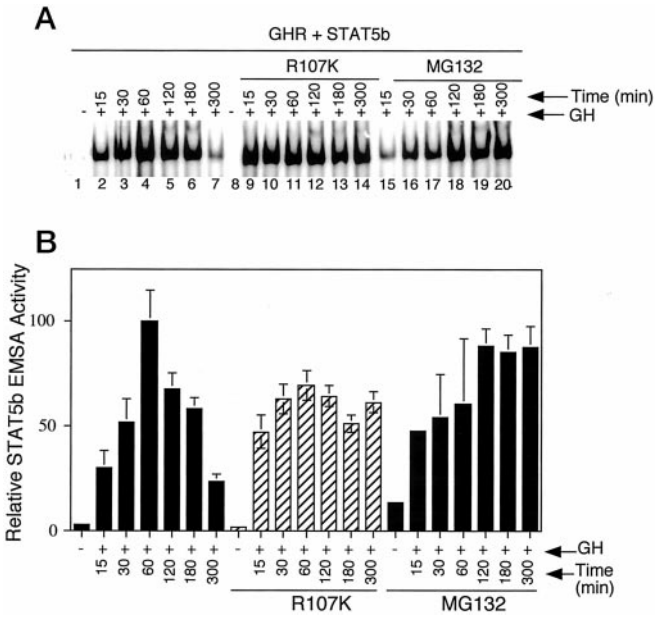


FIG. 6. **CIS mutant R107K abolishes down-regulation of STAT5b signaling.** COS-1 cells transfected with STAT5b (100 ng) and GHR (200 ng) with or without CIS-R107K (500 ng) were treated with GH (200 ng/ml) for times up to 5 h. Cells were treated with 50 μM MG132 for 15 min prior to GH stimulation, as indicated. Extracts were analyzed for STAT5b EMSA activity at each of the indicated time points. Shown is an EMSA gel (*panel A*) with quantitation by PhosphorImager analysis (*panel B*).

naling to STAT5b is ~80–90% down-regulated (18). STAT5b translocation to the nucleus at 45 min is highly efficient in both GFP-transfected cells (Fig. 7, *panel C versus B*) and in untransfected cells growing on the same plate (data not shown). Moreover, the down-regulation of STAT5b signaling after 180 min GH treatment is associated with return of STAT5b to the cytosol (*panel D versus C*). In cells transfected with CIS, GH-stimulated STAT5b nuclear translocation assayed at 45 min was partially inhibited (*c.f.* extent of STAT5b concentration in the nucleus shown in *panel G versus F*), in agreement with the partial inhibition of STAT5b EMSA activity under the same conditions (*e.g.* Fig. 2). In a control experiment, SOCS-3 blocked the GH-stimulated STAT5b translocation seen in untransfected control cells on the same coverslip (*panel K versus J*). Co-transfection of CIS-R107K in combination with CIS (4:1 plasmid weight ratio) blocked the partial inhibitory action of CIS on STAT5b nuclear translocation (data not shown), in agreement with the dominant-negative activity of the CIS mutant described above (Fig. 5). Finally, in cells transfected with CIS-R107K alone, STAT5b translocated to the nucleus at 45 min (*panel O*) and remained nuclear for at least 180 min (*panel Q*). In contrast, in untransfected cells growing on the same coverslip, signaling to STAT5b was efficiently down-regulated by continuous GH, as indicated by the return of STAT5b to the cytosol at 180 min (*panel P versus N*). Given the selectivity of CIS-R107K for the inhibitory action of CIS (Fig. 5, *A* and *B*), the persistence of STAT5b signaling in CIS-R107K-transfected COS-1 cells (Fig. 6) and CWSV-1 cells (Fig. 7) is likely due to CIS-R107K's dominant-negative action toward the endogenous CIS gene product that is induced in response to GH treatment and otherwise serves to down-regulate this signaling pathway (32).

DISCUSSION

Multiple cytokine and growth factor-inducible SOCS/CIS proteins have the potential to inhibit GHR-JAK2 signaling via distinct mechanisms, with SOCS-1 serving as a direct inhibitor of the tyrosine kinase JAK2 (52), and SOCS-3 and CIS exerting their inhibitory action by binding to distinguishable subsets of phosphotyrosine residues in the GHR cytoplasmic domain (32, 38). In the present study, we further elucidate the inhibitory actions of the GH-inducible inhibitor CIS, and we identify several key features that distinguish the inhibitory mechanism of CIS from that of SOCS-1 and SOCS-3. Moreover, we use an SH2 domain mutant of CIS, CIS-R107K, to demonstrate a key role for CIS, but not other GH-inducible SOCS/CIS proteins, in mediating the down-regulation of GHR-JAK2 signaling to STAT5b that occurs in liver cells exposed to GH continuously.

CIS inhibition of GH signaling to STAT5b is presently shown to involve two mechanisms that are distinct but linked: 1) an initial inhibition of GHR-dependent STAT5b activation via an apparent competition between CIS and STAT5b for the same, or perhaps an overlapping set of phosphotyrosine-binding sites on the GHR cytoplasmic tail; and 2) a time-dependent, proteasome-mediated inhibition that involves degradation of CIS, perhaps in association with degradation of the GHR-JAK2 signaling complex. The initial inhibitory action of CIS is manifest immediately upon activation of GHR-JAK2 signaling in the present series of experiments, where COS-1 cells were transiently transfected with CIS cDNA 24 h prior to GH addition. However, in the case of GH-stimulated liver cells, this initial inhibitory phase would not become apparent until ~45–60 min after GH stimulation, at which point GH-stimulated CIS expression is first seen (31, 32). Because this inhibitory mechanism appears to involve competition between CIS and STAT5b for common GHR cytoplasmic tail-binding sites, the extent of CIS inhibition is likely to vary with the cytoplasmic ratio of STAT5b to CIS protein, and as a function of the intracellular concentrations of these proteins relative to that of GHR at the plasma membrane. Indeed, more effective inhibition by CIS is seen in cells transfected with a lower amount of STAT5b, both in the present study (Fig. 1) and in an earlier report (53). Moreover, CIS displayed more extensive inhibition in that earlier study in cells stimulated with lower concentrations of GH (53), a condition that could be expected to yield a lower density of activated, cell surface GHRs, and thus a relative scarcity of GHR cytoplasmic domain-binding sites for CIS and STAT5b. Of note, the inhibitory action of CIS described here has also been observed in CIS transgenic mice in liver *in vivo* (53), but was not seen in transfection studies reported by others (30, 38). This discrepancy might be explained by the expression of comparatively high levels of STAT5b or GHR or by the use of high GH concentrations in those latter studies. The STAT5b dependence of CIS inhibition described here was not seen in the case of SOCS-3 (Fig. 1), a finding that is consistent with SOCS-3 binding primarily to membrane-proximal GHR phosphotyrosine residues, which are distinct from GHR's more COOH-terminal major STAT5b-binding sites (32).

The initial GHR-binding phase of CIS inhibition is followed by a GH-stimulated, time-dependent inhibition of signaling to STAT5b that is proposed to involve proteasome degradation of CIS in association with the GHR-JAK2 signaling complex. Evidence in support of this hypothesis includes our finding that GH stimulated a time-dependent degradation of CIS protein, which could be blocked by the proteasome inhibitor MG132 (Fig. 3). A corresponding degradation was not observed in the case of SOCS-3, whose inhibitory action does not exhibit the time dependence that is characteristic of CIS (Fig. 2). Moreover, proteasome activity is required for the time-dependent inhibitory action of CIS, but not for the inhibitory activity of SOCS-1, SOCS-2, or SOCS-3, as indicated by the ability of MG132 to selectively abrogate the time-dependent component of CIS inhibition. As would be expected, proteasome inhibitor

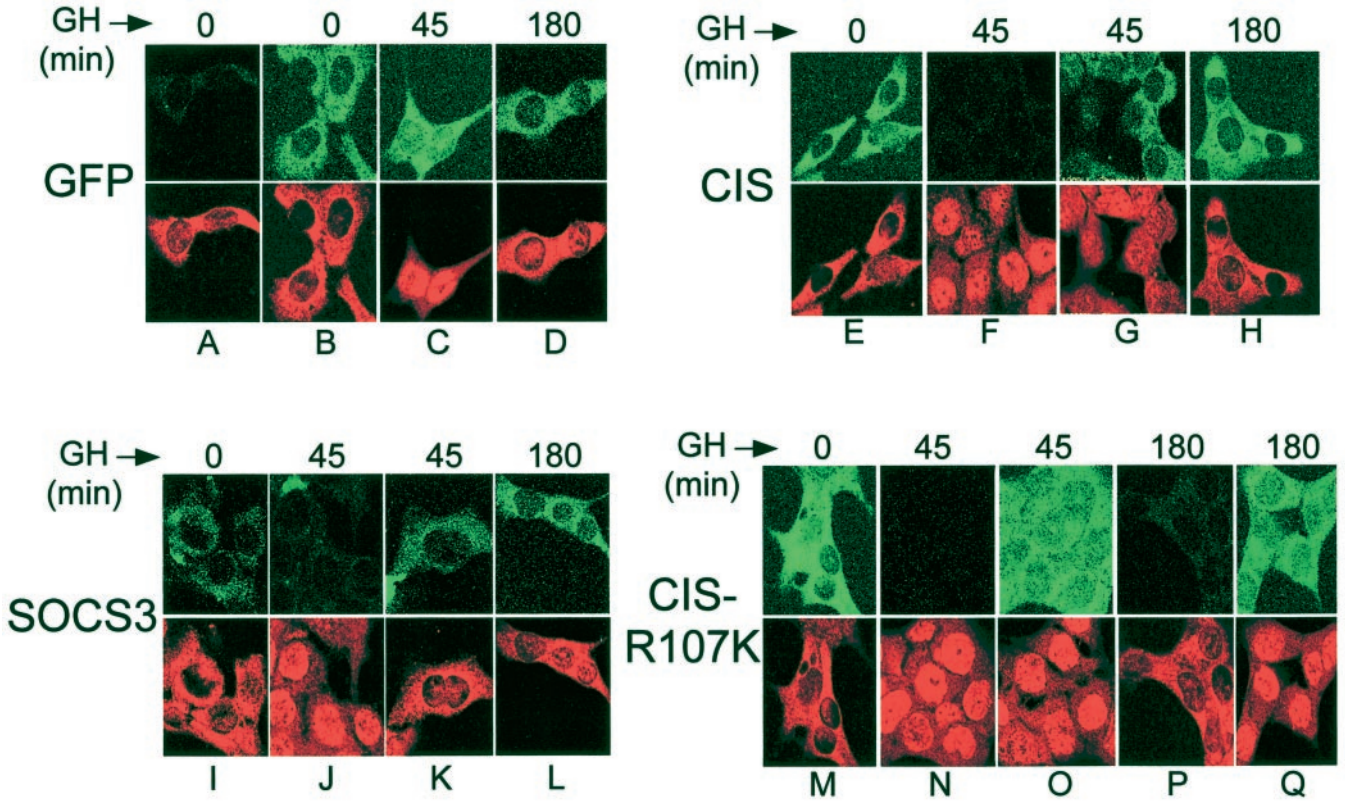
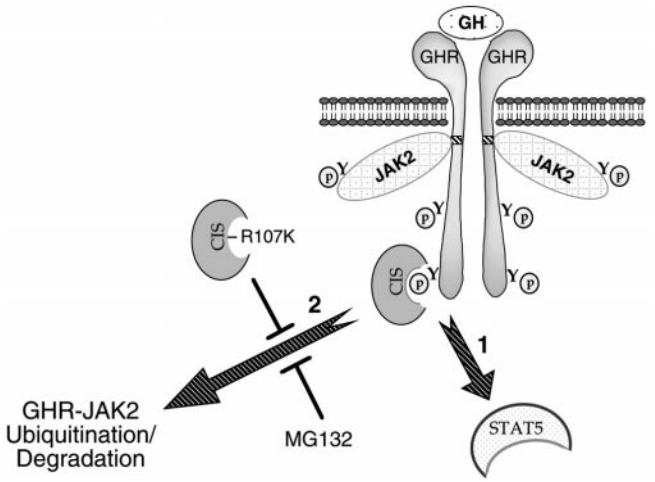


FIG. 7. **Impact of CIS and CIS-R107K on STAT5b nuclear translocation in the liver cell line CWSV-1.** CWSV-1 cells were transfected with GFP alone (*panels A-D*) or GFP in combination with CIS (*panels E-H*), SOCS-3 (*panels I-L*), or CIS-R107K (*panels M-Q*). Cells were stimulated with GH for either 45 or 180 min and the subcelluar localization of STAT5b monitored by indirect immunofluorescence. Shown are confocal microscope images of representative fields revealing the effects of the transfected SOCS/CIS proteins on STAT5b nuclear localization (red fluorescence; *bottom sets of panels*) in response to GH stimulation. Also shown are the corresponding green fluorescent images (*top sets of panels*), which directly identify cells that are transfected with plasmid DNA. Cells shown in *panels A, F, J, N,* and *P* do not efficiently express GFP and are either non-transfected or poorly transfected. *Panels A* and *B, F* and *G, J* and *K, N* and *O*, and *P* and *Q* correspond to images of untransfected and transfected cells, respectively, growing on the same coverslip. Results shown are representative of at least three independent sets of experiments.

had no effect on the initial phase of CIS inhibition seen in cells treated with GH for 30 min (Fig. 4). Further support for the proposed role of CIS in stimulating proteasome-dependent GHR degradation is provided by the finding that GHR undergoes ligand-induced, proteasome-dependent down-regulation via receptor internalization (54). Interestingly, this receptor internalization step does not require GHR ubiquitination (54). This latter finding is consistent with the possibility that CIS protein bound to GHR could serve as the target for ubiquitination, thereby effecting degradation of the CIS·GHR complex. Precedent for such a mechanism is provided by studies of the erythropoietin receptor, where the binding of CIS to a single receptor phosphotyrosine residue is associated with formation of a higher molecular weight, ubiquitinated form of CIS that facilitates receptor degradation, but which accumulates in cells treated with proteasome inhibitor (55). CIS ubiquitination has also been observed in the case of thrombopoietin receptor signaling (56). Our inability to detect a corresponding higher molecular weight form of CIS following GH treatment (data not shown) may reflect a greater instability of ubiquitinated CIS in our system, or alternatively, may result from intrinsic differences in the mechanism whereby CIS stimulates proteasome-dependent down-regulation of GH *versus* erythropoietin and thrombopoietin signaling. Further evaluation of the proposed mechanism of CIS action on GH signaling will require additional study, including an examination the fate of GHR protein in CIS-expressing cells.

The termination of GHR-JAK2 signaling to STAT5b in liver cells at the conclusion of a GH pulse and its down-regulation in cells treated with GH continuously can be blocked by treatment of cells with the proteasome inhibitor MG132 (18, 50). Furthermore, the low-level STAT5b signaling that persists in liver cells exposed to GH chronically is dependent on ongoing protein synthesis and can be augmented in the presence of MG132, suggesting that the GHR·JAK2 signaling complex is labilized under conditions of continuous GH treatment (18). The present finding that CIS mediates a proteasome-dependent inhibitory action on GHR signaling to STAT5b raised the possibility that CIS protein induced by GH treatment could be a key mediator of the down-regulation of GHR-JAK2 signaling in continuous GH-treated cells. Strong support for this hypothesis is now provided by our discovery that CIS-R107K, a dominant-negative CIS mutant (43), can effectively block the continuous GH-induced down-regulation of GHR-JAK2 signaling to STAT5b. This action of CIS-R107K was seen in transfected COS-1 cells (Fig. 6) and also in the liver cell line CWSV-1 (Fig. 7), where all of the major components required for GH-stimulated STAT5b activation and continuous GH-induced down-regulation are expressed endogenously. This striking inhibitory action of CIS-R107K with respect to GH desensitization is presumably a reflection of its dominant-negative action toward the endogenous *CIS* gene, whose expression is induced in CWSV-1 cells within 45 min of GH treatment (32). A similar inhibitory action of the CIS mutant toward endogenous CIS protein has been proposed to account for the enhancement of interleukin 2-stimulated, STAT5-dependent transcription in cells transfected with CIS-R107K (43). Our finding that the negative activity of CIS-R107K is selective for CIS and is not manifest in the case



SCHEME 1. **Model for dual mechanism whereby CIS inhibits GHR-JAK2 signaling to STAT5b.** Step 1 is proposed to involve competition between CIS and STAT5b for common phosphotyrosine-binding site(s) on GHR cytoplasmic tail. Step 2 is proposed to involve CIS-dependent degradation of a CIS-(GHR-JAK2) complex via the proteasome. Step 2 but not step 1 is blocked in cells treated with the proteasome inhibitor MG132 and in cells expressing the CIS SH2 domain mutant CIS-R107K, which does not bind GHR.

of SOCS-1, SOCS-2, or SOCS-3 (Fig. 5) implies that CIS itself, or perhaps a mechanistically similar SOCS/CIS protein, is responsible for the down-regulation of signaling to STAT5b that normally occurs in liver cells stimulated with GH chronically.

The present finding that a single SOCS/CIS protein can mediate the down-regulation of GHR-JAK2 signaling in continuous GH-treated liver cells is somewhat unexpected, in view of reports demonstrating that GH can induce multiple SOCS/CIS mRNAs in cultured hepatocytes and in rodent liver *in vivo* (30, 31). Strong down-regulation of STAT5b signaling by continuous GH, such as that seen in cultured liver cells (18, 46), typifies adult female rat liver, which is exposed to plasma GH in a near-continuous manner, resulting in a level of activated, nuclear STAT5 that is on average only ~10% that observed in adult male rats during a plasma GH pulse (15, 17). Accordingly, CIS is proposed to be a major factor responsible for the down-regulation of STAT5b (and STAT5a) signaling in female rat liver *in vivo*. Indeed, CIS mRNA levels are ~2-fold higher in female compared with male rat liver according to one study (34). CIS may be well suited for this task, insofar as liver CIS mRNA persists for several hours following exogenous hormone treatment, both in the case of GH (32, 34) and prolactin stimulation (35).

An intact SH2 domain is required for CIS to inhibit cytokine and growth factor receptor signaling pathways. Accordingly, mutation of the conserved phosphotyrosine-binding FLVR motif within CIS's SH2 domain yields a protein, CIS-R107K, that is devoid of inhibitory activity toward interleukin-2 receptor signaling and was proposed to exert dominant-negative activity toward an endogenous wild-type CIS (43). This dominant-negative activity of CIS-R107K was verified and further characterized in the present study of GH-stimulated STAT5b signaling. The mechanism for this dominant-negative action of CIS-R107K is intriguing. In contrast to CIS, CIS-R107K did not bind to tyrosine-phosphorylated GHR expressed as a fusion protein (Fig. 5*D*) and, correspondingly, did not significantly inhibit GHR-JAK2 signaling. Although *in vitro* binding interactions may not necessarily be fully predictive of the situation in intact cells, these findings nonetheless point to the importance of an intact CIS SH2 domain, both for GHR binding and for inhibition of STAT5b signaling. In contrast, in the case of the interleukin-2 receptor, mutation of the CIS SH2 domain abolishes the inhibition of STAT5b signaling but not receptor binding (43). Despite its apparent inability to bind GHR, CIS-R107K effectively blocked the time-dependent component of wild-type CIS inhibition that is associated with proteasome activity (Fig. 5, *A* and *B*). This finding suggests that CIS-R107K may serve as a "decoy" that spares wild-type CIS protein and the associated GHR-JAK2 signaling complex from proteasome-dependent degradation (Scheme 1). In contrast, CIS-R107K did not exert dominant-negative activity toward the GHR binding/STAT5b competition component of CIS inhibition (Fig. 5*E*), as would be expected based on CIS-R107K's inability to bind directly to GHR. Corresponding SH2 domain mutants of SOCS-1 (SOCS-1-R107K and SOCS-1-R105E), while inactive with respect to inhibition of GHR signaling to STAT5b (also see Refs. 57 and 58)), failed to exhibit SOCS-1 dominant-negative activity (data not shown). This latter observation provides further support for the unique inhibitory mechanism of CIS highlighted by the present study. Proteasome-linked regulatory mechanisms have been postulated for SOCS-1 and SOCS-3, and in both cases association of the SOCS protein with the ubiquitin-like molecule elongin B has been reported (59, 60). The absence of an effect of proteasome inhibitor on SOCS-1 and SOCS-3 inhibited GH signaling (Fig. 4) suggests, however, that in contrast to CIS, proteasome action is not an essential step in the GH signal inhibitory activity of these latter two SOCS proteins.

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