Signal transducers and activators of transcription (STAT) factors are cytoplasmic proteins that induce gene activation in response to cytokine receptor stimulation. Following tyrosine phosphorylation, STAT proteins dimerize, translocate into the nucleus, and activate specific target genes. Activation is transient, and down-regulation of STAT signaling occurs within a few hours. In the present study, we show that the cyclin-dependent kinase inhibitor \( p21^{WAF1/CIP1/SDI1} \) inhibits STAT3 transcriptional activation. Following leukemia inhibitory factor stimulation, \( p21^{WAF1/CIP1/SDI1} \) was found to associate with STAT3 proteins in communoprecipitation and pull down assays. In vivo, overexpression of \( p21^{WAF1/CIP1/SDI1} \) reduced transcriptional activation by STAT3 proteins but did not modify DNA binding activity. Interestingly, pull down experiments showed that \( p21^{WAF1/CIP1/SDI1} \) could interact with the CREB-binding coactivator protein, and inhibition of STAT3 activity by \( p21^{WAF1/CIP1/SDI1} \) did not occur when CREB-binding protein was overexpressed. These results suggest a model by which \( p21^{WAF1/CIP1/SDI1} \) functions as an inhibitor of STAT3 signaling and highlight a new activity for this cyclin-dependent kinase inhibitor.

Proliferation and cellular differentiation are regulated by secreted proteins known as cytokines. Based on their structural similarities and shared use of gp130 receptor subunit, interleukin (IL) 6, the leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor, interleukin 11 (IL-11), and cardiotrophin-1 define the IL-6-type cytokine family (1). These cytokines exert multiple functions on cell growth and differentiation, such as activation of hepatocyte transcription, activation of neural proliferation and differentiation, and regulation of hematopoiesis. Moreover, LIF, ciliary neurotrophic factor, cardiotrophin-1, and oncostatin M display biological properties in the early stages of embryonic development (2–3).

Binding of these cytokines to their receptors activates the JAK protein tyrosine kinases, followed by tyrosine phosphorylation of the receptors. This leads to activation and homo- or heterodimerization of the STAT3/3 transcription factors, translocation into the nucleus, and activation of target genes (4–5). This activation is transient, and activated transcription factors disappear from the nucleus within 1–6 h after ligand stimulation (4, 6). This suggests that inhibitory mechanisms should exist, and several pathways leading to the inhibition of receptor signaling and STAT function have been recently described. Suppressor of cytokine signaling and CIS/JAB proteins were originally discovered on the basis of their ability to interact with and inhibit the tyrosine phosphorylation of the JAK tyrosine kinase and to compete with STAT proteins for binding to phosphotyrosine residues within the cytoplasmic domains of cytokine receptors (7–8). Similarly, the protein tyrosine phosphatase SHP-2 has been shown to be recruited to cytokine receptor to attenuate gp130-mediated signaling by modulating JAK activation (9). Removal of activated STAT proteins from the nucleus has been shown to require phosphatase action, with reappearance of the STAT in the cytoplasm in a dephosphorylated state (10–11). Others experiments have also indicated an important role for proteolytic degradation in this inhibitory pathway (12). Phosphorylated STAT proteins have been detected in association with ubiquitin, whereas proteasome inhibitors prolong the activation of these transcription factors (12). Association with inhibitory molecules has also been recently described; the PIAS family of proteins was identified as negative regulators of STAT activity (13). Association between PIAS and STAT proteins occurs after cytokine stimulation and requires tyrosine phosphorylation of the transcription factor. PIAS proteins have been shown to inhibit DNA binding, but the precise mechanism of STAT down-modulation remains to be clarified. Interestingly, these experiments have suggested the involvement of specific PIAS protein in the STAT1 and STAT3 signaling pathways (14). Additional stimuli can also block the JAK-STAT signaling cascade, suggesting that there are several ways for limiting the strength and duration of cytokine signaling (15).

Mitogenic stimulation leads to the transition from G1 to S phase of the cell cycle through the synthesis of proteins termed cyclins (16). This progression is thought to be regulated by the periodic activation of complexes of cyclin-dependent kinases (cdks). Cdks are activated by cyclin association, phosphorylation by cdk-activating kinase, and association with cdk inhibitors such as \( p21^{WAF1/CIP1/SDI1} \) (17–19). \( p21^{WAF1/CIP1/SDI1} \) was originally isolated as a transcriptional target of p53 (20), and it is now known that this protein is a component of a complex containing cyclins, CDks, and proliferating cell nuclear antigen (21–22). An increased amount of \( p21^{WAF1/CIP1/SDI1} \) in this quaternary complex leads to an inhibition of DNA synthesis and cell cycle arrest (23), which finally facilitate DNA repair processes. Consequently, expression of the \( p21^{WAF1/CIP1/SDI1} \) protein is induced by several proteins that inhibit cell cycle progression, including p53,
transformation growth factor β, MyoD, and CCAAT/enhancer-binding protein α (20, 24–26). However, the biological role of the p21-cyclin-cdk association remains to be clarified. p21<sub>WAF1/CIP1/SDI1</sub> expression is induced when quiescent cells are stimulated to proliferate (27), whereas the majority of cdk complexes in proliferating cells have been reported to interact with p21<sub>WAF1/CIP1/SDI1</sub> (28). Recent results have shown that p21<sub>WAF1/CIP1/SDI1</sub> promotes the assembly of active cyclin D-cdk4 complexes, suggesting a new role for this inhibitor as an adapter protein (29). Several observations raised the possibility that p21<sub>WAF1/CIP1/SDI1</sub> has roles at other stages of the cell cycle or during cell differentiation. p21<sub>WAF1/CIP1/SDI1</sub> accumulates near the G<sub>S</sub>/M boundary and contributes to the onset of mitosis by facilitating the implementation of G<sub>S</sub>/M checkpoint controls (30). p21<sub>WAF1/CIP1/SDI1</sub> was also shown to play a positive role in the commitment to differentiate, as this protein is up-regulated in the early stage of differentiation (25, 31). However, this level is decreased at the late stages, and forced expression of p21<sub>WAF1/CIP1/SDI1</sub> inhibited the differentiation of keratinocytes (32), suggesting that p21<sub>WAF1/CIP1/SDI1</sub> may also play inhibitory roles in this program. Finally, other results indicate new biochemical activities for this protein. p21<sub>WAF1/CIP1/SDI1</sub> has also been shown to interact with and stimulate NF-κB-dependent gene expression through inhibition of CBP/p300-associated cyclin E-Cdk2 activity (34). Altogether, these data indicate that p21<sub>WAF1/CIP1/SDI1</sub> could be considered as a bridge between signaling complexes involved in cell cycle, tumor suppression, senescence, and cellular stress.

STAT proteins can recognize a conserved element in the promoter of p21<sub>WAF1/CIP1/SDI1</sub> and increase the expression of this gene (35–36). Thus, p21<sub>WAF1/CIP1/SDI1</sub> may be one of the target genes activated by the JAK-STAT cascade; however, the potential role of this protein in this signaling pathway remains to be determined. In this study, we show that following LIF stimulation, p21<sub>WAF1/CIP1/SDI1</sub> interacts with STAT3 proteins and inhibits the transcriptional activity of these factors. These results reveal that p21<sub>WAF1/CIP1/SDI1</sub> is part of a feedback network controlling the down-modulation of STAT activity.

MATERIALS AND METHODS

Cell Culture and Reagents—The TF1 erythroleukemia, HepG2 hepatoma, and COS cell lines obtained from the American Type Culture Collection (Manassas, VA) were grown in RPMI medium supplemented with 10% fetal calf serum or in RPMI 10% fetal calf serum supplemented with 1 ng/ml GM-CSF for TF1 cells. When required, cells were serum-starved for 1–3 days to obtain cells in the G<sub>0</sub> phase of the cell cycle. Purified recombinant cytokines were obtained from Dr. K. Turner (Genetics Institute, Boston). Vectors expressing p53<sub>WAF1/CIP1/SDI1</sub> CBP, and GST-p21<sub>WAF1/CIP1/SDI1</sub> could be obtained as gifts of Dr. M. Roussel, Dr. X. Y. Yang, and Dr. R. Fotedar, respectively. Plasmids expressing the gp130 and LIF receptor X. Y. Yang, and Dr. R. Fotedar, respectively. Plasmids expressing the gp130 and LIF receptor X. Y. Yang, and Dr. R. Fotedar, respectively. Plasmids expressing the gp130 and LIF receptor X. Y. Yang, and Dr. R. Fotedar, respectively. Plasmids expressing the gp130 and LIF receptor X. Y. Yang, and Dr. R. Fotedar, respectively. Plasmids expressing the gp130 and LIF receptor X. Y. Yang, and Dr. R. Fotedar, respectively. Plasmids expressing the gp130 and LIF receptor X. Y. Yang, and Dr. R. Fotedar, respectively.

Interaction between p21<sub>WAF1/CIP1/SDI1</sub> and STAT3 Proteins—To assess the ability of p21<sub>WAF1/CIP1/SDI1</sub> to interact with the STAT-signaling pathway, experiments were carried out on the human HepG2 hepatoma cell line expressing the LIF receptor transducing complex. STAT3 DNA binding activity was induced by LIF stimulation in this cell line, reached its maximum after 1 h, and then gradually decreased (Fig. 1, lane 2). EMSA experiments were conducted in the presence of antibodies against STAT3 to prove that the binding was specific for this protein (Fig. 1, lane 3). Western blot analysis was performed to determine the activation of STAT3 after stimulation (Fig. 1, lanes 9–13). Taken together, these results suggest that STAT3 activity is down-modulated in HepG2 after LIF stimulation and that STAT3 activation might be involved in the regulation of STAT3 activation, we first asked whether it could bind specifically to STAT3 proteins. Following LIF stimulation, nuclear extracts were recovered from HepG2 cells, and coimmunoprecipitations were performed alternatively with monoclonal antibodies directed against p21<sub>WAF1/CIP1/SDI1</sub> or polyclonal antibodies directed against STAT3, and proteins present in the immunoprecipitates were revealed by immunoblotting with the

RESULTS

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Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts prepared as described above were preincubated for 5 min at room temperature in 25 mM NaCl, 10 mM Tris, pH 7.5, 1 mM MgCl<sub>2</sub>, 5 mM EDTA, pH 8, 5% glycerol, and 1 mM dithiothreitol with 1 μg of poly(dI-dC) as a nonspecific competitor. Where indicated, extracts were preincubated for 1.5 h at 4 °C with 1 μg of polyclonal antibodies (C20) directed against STAT3 in the presence of 1% Brij 96. A double-stranded nucleotide containing a Stat3-consensus binding site derived from the c-fos gene (the inducing element) was added and labeled using the T4 kinase, and 10 pg of probe (20,000 cpm) was added. The probe was incubated for 15 min on ice, then annealed on a 5% polyacrylamide gel (30.1) and separated by electrophoresis in 50 mM Tris, 0.38 mM glycine, 1 mM EDTA, pH 8.5. Gels were then dried and visualized by autoradiography.

Immunoprecipitation and Western Blot Analysis—Nuclear cell extracts (1–5 μg) were recovered as described above, and immunoprecipitations were then performed in the presence of 1% Brij 96 with the indicated antibodies overnight at 4 °C on a rotator. Following a 30-min incubation at 4 °C, nuclear extracts were spun down at 12,000 rpm for 5 min. Extracts were either used immediately or frozen and stored at −80 °C. For total cell extracts, 200 μl of extract (10 μg of protein) was added to the plates. After 15 min incubation, total extracts were recovered by centrifugation at 12,000 rpm for 10 min, and extracts were either used immediately or frozen and stored at −80 °C.
Regulation of STAT3 Activation by p21WAF1/CIP1/SDI1

Fig. 1. EMSA analysis showing STAT3 DNA binding following LIF stimulation. HepG2 cells were serum-starved for 48 h and stimulated by adding fresh RPMI medium with LIF (20 ng/ml). The indicated time corresponds to the time elapsed since cytokine stimulation. Nuclear extracts were prepared, and 5 μg were incubated at room temperature for 15 min with radiolabeled oligonucleotides encoding a STAT3 consensus binding site, in the presence or in the absence of polyclonal antibodies directed against STAT3 proteins (lane 8) or a nonimmune serum (lane 7). DNA-protein complexes were then resolved on a nondenaturing polyacrylamide gel. Proteins from the same extracts were separated by SDS-PAGE electrophoresis, blotted, and probed with a phospho-STAT3-Tyr705-specific antibody (lanes 9–13, top part) or a polyclonal antibody directed against STAT3 (lanes 9–13, bottom part).

Fig. 2. In vivo association between p21WAF1/CIP1/SDI1 and STAT3 proteins. A, HepG2 cells were serum-starved for 48 h and then stimulated with LIF (20 ng/ml) for the indicated times. Nuclear cell extracts (5 mg) were immunoprecipitated (IP) with monoclonal antibodies directed against p21WAF1/CIP1/SDI1 proteins, separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with polyclonal antibodies directed against STAT3 proteins (lanes 1–4). Reciprocal immunoprecipitations were performed with polyclonal antibodies directed against STAT3 proteins followed by membranes blotting with polyclonal antibodies directed against p21WAF1/CIP1/SDI1 proteins (lanes 5–8). B, TF1 cells were serum-starved for 24–48 h and then stimulated with LIF (20 ng/ml) for the indicated times. Immunoprecipitations were performed as described in A. C, TF1 cells were stimulated for 4 h with LIF (20 ng/ml, lanes 2 and 5) and GM-CSF (10 ng/ml, lane 4), and nuclear extracts were immunoprecipitated with monoclonal antibodies directed against p21WAF1/CIP1/SDI1 proteins and probed with polyclonal antibodies directed against STAT5b (lanes 1 and 2) or STAT3 (lanes 3–5) proteins. D, HepG2 cells were serum-starved for 48 h and then stimulated with LIF (20 ng/ml) for 2 h. Nuclear extracts were prepared, and extracts (1 mg) were either left untreated (lane 2) or sequentially depleted by three rounds of immunoprecipitation with either polyclonal antibodies directed against the CBP (lane 1), STAT3 proteins (lane 5), or monoclonal antibodies directed against the IL-6 receptor gp130 (lane 3) or p21WAF1/CIP1/SDI1 (lane 6). A nonimmune mouse serum was used as a negative control (lane 4). Following depletion, supernatants were immunoprecipitated with monoclonal antibodies directed against STAT3 proteins, separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with polyclonal antibodies directed against STAT3 proteins.

To confirm and extend these results, p21WAF1/CIP1/SDI1 proteins were depleted from nuclear extracts of LIF-stimulated HepG2 cells by three sequential rounds of immunoprecipitations. Depleted lysates were then precipitated and blotted with anti-STAT3 antibodies, in order to estimate the STAT3 residual level that remained unassociated with p21WAF1/CIP1/SDI1 proteins (Fig. 2D). Three rounds of immunodepletion with p21WAF1/CIP1/SDI1 antibodies removed the majority of STAT3 proteins from lysates of induced HepG2 cells, indicating that a substantial fraction of STAT3 proteins is associated with p21WAF1/CIP1/SDI1 following LIF stimulation (Fig. 2D, compare lanes 2–4 and 6). As described previously, STAT3 proteins also coprecipitated with the CREB-binding protein (CBP) (Fig. 2D, compare lanes 1 and 2), with antibodies to STAT3 (Fig. 2D, compare lanes 2 and 5), but no depletion was observed when a nonimmune mouse serum or antibodies directed against the gp130 cytokine receptor were used (Fig. 2D, lanes 2–4).

Altogether, these results indicate that STAT3 signaling proteins interact with p21WAF1/CIP1/SDI1.
Regulation of STAT3 Activation by p21WAF1/CIP1/SDI1

Fig. 3. Effect of p21WAF1/CIP1/SDI1 on STAT3 DNA binding activity. A, HepG2 cells (lanes 1–3) were transfected with empty vectors (lane 1) and vectors expressing p21WAF1/CIP1/SDI1 (lane 3) or STAT3 proteins (lanes 2 and 3). Following transfection, cells were serum-starved for 48 h and stimulated for 1 h with LIF (20 ng/ml; lanes 2 and 3). Nuclear extracts were then prepared and incubated for 15 min at room temperature with radiolabeled oligonucleotides encoding a STAT3 consensus binding site. B, COS cells were transfected with plasmids expressing the gp130 and LIF receptor β-transduction unit, together with empty vectors (lanes 1, 4, and 9) or vectors expressing STAT3 (lanes 2 and 3, 5 and 6, and 10 and 11) or p21WAF1/CIP1/SDI1 (lanes 3, 6, and 11). Following transfection, cells were serum-starved for 48 h and then stimulated with LIF (20 ng/ml) for 1 h. Nuclear extracts were then analyzed by EMSA (lanes 1–6) or Western blot using monoclonal antibodies directed against STAT3. C, COS cells (lanes 7 and 8) were serum-starved for 48 h and then stimulated with LIF (20 ng/ml) for 1 h. Nuclear cell extracts (1 mg) were immunoprecipitated (IP) with monoclonal antibodies directed against p21WAF1/CIP1/SDI1 proteins, separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with polyclonal antibodies directed against STAT3 proteins (lanes 1 and 2). C, COS cells were transfected with vector DNA expressing the gp130 and LIF receptor β-transduction unit, together with empty vectors (lanes 1 and 2) or STAT3 (lanes 3 and 4). Western blot experiments using nuclear extracts showed that the steady-state level of STAT3 proteins did not decrease in the presence of p21WAF1/CIP1/SDI1 (Fig. 3B, lane 3 and 4), further indicating that the inhibitory effect was shared by cytokines using the gp130 and STAT3 signaling pathways. Importantly, no effect of p21WAF1/CIP1/SDI1 on the activation of a Gal4-VP16 fusion protein (Fig. 4C, compare lanes 3 and 4), or on the activation of a Gal4-VP16 fusion protein (Fig. 4C, compare lanes 6 and 7). Western blot experiments using nuclear extracts showed that the steady-state level of STAT3 proteins did not decrease in the presence of p21WAF1/CIP1/SDI1 (Fig. 4D, lanes 1 and 2, see also Fig. 3C), further indicating that the varying level of transcriptional activity was not a result of differences in protein stabilities. Altogether, these results suggest that p21WAF1/CIP1/SDI1 could inhibit the transcriptional activity of STAT3. To verify this, HepG2 cells were cotransfected with a reporter construct containing three STAT3 consensus binding sites upstream of a thymidine kinase minimal promoter, together with vectors expressing STAT3 and p21WAF1/CIP1/SDI1. Following transfection, cells were serum-starved for 15 h and stimulated with LIF, and luciferase activity was measured after 15 h on cytoplasmic extracts. Inclusion of a STAT3-expressing vector in the transfection mix led to a 6-fold increase in expression following cell stimulation (Fig. 4A, lanes 1 and 2). Activation by STAT3 was completely abolished in the presence of a p21WAF1/CIP1/SDI1 expression vector (Fig. 4A, compare lanes 2 and 3). This effect was also observed when STAT3 expression vectors were omitted from the transfection mix, suggesting that p21WAF1/CIP1/SDI1 was able to block the activation of the endogenous STAT3 proteins (data not shown). p21WAF1/CIP1/SDI1 was also able to block the transcriptional activation induced by IL-6 (Fig. 4B, compare lanes 2 and 3) and oncostatin M (data not shown), indicating that p21WAF1/CIP1/SDI1 inhibitory effect was shared by cytokines using the gp130 and STAT3 signaling pathways. Importantly, no effect of p21WAF1/CIP1/SDI1 was observed on the spontaneous expression of the reporter gene (data not shown), on a minimal thymidine kinase promoter (Fig. 4C, compare lanes 3 and 4), or on the activation of a Gal4-VP16 fusion protein (Fig. 4C, compare lanes 6 and 7). Western blot experiments using nuclear extracts showed that the steady-state level of STAT3 proteins did not decrease in the presence of p21WAF1/CIP1/SDI1 (Fig. 4D, lanes 1 and 2, see also Fig. 3C), further indicating that the varying level of transcriptional activity was not a result of differences in protein stabilities.
inhibit the transcriptional activity of STAT3 proteins.

Role of the CREB-binding Protein CBP in p21<sup>WAF1/CIP1/SDI1</sup>-mediated Inhibition of STAT3 Activity—Transcriptional activation by STAT proteins has been shown to require interactions with the coactivator CREB-binding protein (CBP) (39–42). Given that STAT3 proteins appeared to be targets for transcriptional repression by p21<sup>WAF1/CIP1/SDI1</sup>, we sought to investigate the possibility of a functional cross-talk between p21<sup>WAF1/CIP1/SDI1</sup>, STAT3 proteins, and CBP. We first asked whether CBP and p21<sup>WAF1/CIP1/SDI1</sup> could interact together. To this end, GST or GST-p21<sup>WAF1/CIP1/SDI1</sup> fusion proteins bound to glutathione-Sepharose beads were incubated with HepG2 nuclear extracts. Beads were then extensively washed, and retained proteins were analyzed by immunoblotting with polyclonal antibodies specific for CBP. Under these conditions, CBP was retained by the GST-p21<sup>WAF1/CIP1/SDI1</sup> fusion protein but not by GST alone (Fig. 5A, lanes 1 and 2, top). As expected from the results presented above, STAT3 also bound to GST-p21<sup>WAF1/CIP1/SDI1</sup> but not to GST alone (Fig. 5A, lanes 1 and 2, bottom). Although not proven, these results suggest that p21<sup>WAF1/CIP1/SDI1</sup>, STAT3, and CBP proteins could form a ternary complex.

To confirm and extend these results, p21<sup>WAF1/CIP1/SDI1</sup> proteins were depleted from HepG2 nuclear extracts by three sequential rounds of immunoprecipitations. Depleted lysates were then precipitated and blotted with antibodies to CBP in order to estimate the residual level of CBP that remained unassociated with p21<sup>WAF1/CIP1/SDI1</sup> proteins (Fig. 5B). Immunodepletion with p21<sup>WAF1/CIP1/SDI1</sup> antibodies completely removed CBP proteins from lysates of induced HepG2 cells, indicating that CBP proteins are associated with p21<sup>WAF1/CIP1/SDI1</sup> (Fig. 5B, compare lanes 2–4 and 6). As described above, CBP proteins also coprecipitated with STAT3 (Fig. 5B, lane 1), with antibodies to CBP (Fig. 5B, lane 5), but no significant depletion was observed with antibodies to the gp130 cytokine receptor or with a nonimmune mouse serum (Fig. 5B, lanes 2–4).

Given that CBP low levels are often rate-limiting for STAT-mediated transcription, inhibition of STAT3 activity could potentially result from the interaction between CBP and p21<sup>WAF1/CIP1/SDI1</sup>. We therefore examined the blocking effect of p21<sup>WAF1/CIP1/SDI1</sup> in the presence or absence of coexpressed CBP. STAT3-induced reporter activity was determined following LIF stimulation of HepG2 cells transfected as described above. Consistent with our hypothesis, cotransfection of a CBP expression plasmid abolished the inhibitory effect of p21<sup>WAF1/CIP1/SDI1</sup> on STAT3 activity (Fig. 5C, compare lanes 3 and 4).

DISCUSSION

STAT activation is transient and declines within a few hours following cytokine receptor activation. Multiple levels of regulation have been recently described that act upstream or at the level of STAT proteins. Dephosphorylation, proteolytic degradation, or inhibition by the PIAS family of proteins have been shown to participate in the negative regulation of STAT activation (10–14). The results presented in this study describe a new pathway for inhibiting STAT3 activation that is mediated by the cell cycle inhibitor p21<sup>WAF1/CIP1/SDI1</sup>. We have shown that p21<sup>WAF1/CIP1/SDI1</sup> can interact with STAT3 proteins and block their transcriptional activity. These results point to a novel biological role for p21<sup>WAF1/CIP1/SDI1</sup> in the feedback regulation of IL-6-type cytokine signaling pathways and indicate that gene activation by STAT3 proteins are affected by signals that control cell cycle progression.

Several lines of evidence support a role for p21<sup>WAF1/CIP1/SDI1</sup> in inhibition of cytokine signaling. Activation of STAT factors leads to transcriptional activation of the p21<sup>WAF1/CIP1/SDI1</sup> gene and promotes protein synthesis within a few hours of stimulation (35, 43). p21<sup>WAF1/CIP1/SDI1</sup> was found to be induced when cells are stimulated to proliferate by IL-2, IL-4, or IL-6 (27, 35, 44). These cytokines allow the transition from quiescence to S phase by causing the elimination of the related
feedback loop to regulate cytokine signal transduction and down-modulates signaling through transcriptional inhibition of STAT proteins. Interestingly, transforming growth factor-β has been shown to inhibit IL-2 signaling without having any effect on STAT5 DNA binding activity (45). As transforming growth factor-β up-regulates the expression of p21WAF1/CIP1/SDI1 (46, 47), it may induce an interaction between STAT5 and the p21WAF1/CIP1/SDI1 inhibitor, leading to transcriptional repression of the protein. This hypothesis is also confirmed by recent experiments showing that the p53 tumor suppressor gene inhibits the transcriptional activation of STAT5 proteins (48). Interestingly, this inhibition does not rely on a decrease of the cellular concentration of STAT5 or on interference with DNA binding activity. Since p21WAF1/CIP1/SDI1 was originally isolated as a transcriptional target of p53 (20), it might be tempting to speculate that p53 inhibits STAT5 activation through activation of the p21WAF1/CIP1/SDI1 promoter. Indeed, preliminary experiments done in the laboratory indicate that p21WAF1/CIP1/SDI1 and STAT5 could interact following GM-CSF stimulation of TF1 cells.

The domain(s) that modulates the interaction between p21WAF1/CIP1/SDI1 and STAT3 proteins remains(s) to be identified. It is unlikely that this interaction involves the DNA binding domain of STAT3, since p21WAF1/CIP1/SDI1 does not interfere with DNA binding. Several other regions of STAT proteins have been previously implicated in transcriptional regulation; inactivation by tyrosine phosphatases requires the amino-terminal domain, whereas ubiquitination is dependent on the carboxyl-terminal part of STAT proteins, suggesting that either domain could be involved in the effect of p21WAF1/CIP1/SDI1 (49–51). Depending on the region of interaction, a few possibilities can be raised concerning the molecular mechanisms whereby p21WAF1/CIP1/SDI1 inhibits STAT3 signaling. The amino-terminal region of STAT proteins is involved in dimer-dimer interactions leading to cooperative DNA binding (52, 53). Interaction of p21WAF1/CIP1/SDI1 with this domain may inhibit cooperative DNA binding and prevents transcriptional activation. Interestingly, this amino-terminal domain is conserved among STAT proteins (4), suggesting that it plays an important role in the regulation of these factors. An alternative hypothesis would be that the cdk inhibitor interacts with the activation domain present at the carboxyl-terminal part of the protein. In this model, p21WAF1/CIP1/SDI1 would block the interactions with the RNA polymerase II transcriptional machinery (54, 55). In that case, transcriptional inhibition would be mediated through repression of protein-protein interactions. In line with this hypothesis, the Stat3 carboxyl terminus was recently shown to be capable of recruiting the CBP coactivator (56). CBP promotes the interaction between transcriptional activators and the transcription complex (57). It also contains histone acetyltransferase function of CBP, since it has been shown to stimulate NF-κB gene activation through its interactions with this adapter (34).

An alternative hypothesis would be that STAT3 transcrip-

cdk inhibitor p21Kip1 but also surprisingly induce an increase in the expression of p21WAF1/CIP1/SDI1. This apparent paradox could be resolved if p21WAF1/CIP1/SDI1 acts in a classic negative role in the regulation of these factors. An alternative hypothesis would be that the cdk inhibitor interacts with the activation domain present at the carboxyl-terminal part of the protein. In this model, p21WAF1/CIP1/SDI1 would block the interactions with the RNA polymerase II transcriptional machinery (54, 55). In that case, transcriptional inhibition would be mediated through repression of protein-protein interactions. In line with this hypothesis, the Stat3 carboxyl terminus was recently shown to be capable of recruiting the CBP coactivator (56). CBP promotes the interaction between transcriptional activators and the transcription complex (57). It also contains histone acetyltransferase activity that directly enhances the access of DNA-binding factor to chromatin structure (58–60). Interestingly, recent results have shown that gene activation by STAT proteins requires CBP and histone acetyltransferase activity (39–42). Our results indicate that the CBP coactivator can interact with and block the inhibitory effect of p21WAF1/CIP1/SDI1 on STAT3 functions. It is thus tempting to speculate that p21WAF1/CIP1/SDI1 binds to the carboxyl-terminal part of STAT3 proteins, prevents the interaction with CBP, and inhibits the transcriptional activity of these factors. Importantly, p21WAF1/CIP1/SDI1 does not inhibit the intrinsic histone acetyltransferase function of CBP, since it has been shown to stimulate NF-κB gene activation through its interactions with this adapter (34).

An alternative hypothesis would be that STAT3 transcrip-

FIG. 5. Effect of CBP on the p21WAF1/CIP1/SDI1-dependent inhibition of STAT3 activity. A, HepG2 cells were serum-starved for 48 h and stimulated with LIF (20 ng/ml) for 2 h. Nuclear extracts (1 mg) were then incubated for 1 h at 4 °C with GST alone (lane 1) or GST-p21WAF1/CIP1/SDI1 (lane 2) on glutathione beads. Samples were washed three times and separated by SDS-PAGE on 6% polyacrylamide gels. Membranes were then blotted with polyclonal antibodies directed against CBP (top) or STAT3 proteins (bottom). B, HepG2 cells were serum-starved for 48 h and then stimulated with LIF (20 ng/ml) for 2 h. Nuclear extracts were prepared, and extracts (1 mg) were either left untreated (lane 2) or subjected to three rounds of immunodepletion with either polyclonal antibodies directed against CBP proteins (lane 1) or CBP (lane 5), or monoclonal antibodies directed against the IL-6 receptor gp130 (lane 3) or p21WAF1/CIP1/SDI1 (lane 6). A nonimmune mouse serum was used as a negative control (lane 3). Following depletion, supernatants were immunoprecipitated with polyclonal antibodies directed against CBP proteins, separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with polyclonal antibodies directed against CBP. C, HepG2 cells were cotransfected with a reporter plasmid C, HepG2 cells were cotransfected with a reporter plasmid WAF1/CIP1/SDI1, thymidine kinase.
Regulation of STAT3 Activation by p21WAF1/CIP1/SDI1

tional activity is controlled by proteins that are inhibited by p21WAF1/CIP1/SDI1, e.g. cyclins-cdk complexes. p21WAF1/CIP1/SDI1 has been previously shown to block the activity of cyclin Dcdk4, cyclin Ecdk2, and cyclin Acdk2, all complexes that could therefore regulate STAT3 activity (29). In line with this hypothesis, specific cyclin-dependent kinases can block the activity of different transcription factors such as NF-kB (34). However, cyclins E and A are expressed at the end of the G1 phase of the cell cycle, when STAT proteins are already inactivated (16). These observations would argue that cyclins E and A are probably not necessary for STAT3 transcriptional activity. By contrast, cyclin D expression correlates with STAT3 activation (61) and is induced following cytokine stimulation (62, 63). Therefore, a role for cyclin D in STAT3 regulation seems plausible, and this hypothesis is currently under investigation in the laboratory.

Altogether, these results point to a novel biological role for p21WAF1/CIP1/SDI1 proteins and have uncovered an important component of STAT3 transcriptional regulation. This leads us to propose a model by which cytokine stimulation induces the expression of p21WAF1/CIP1/SDI1, which then interacts with STAT3 proteins and prevents the binding of the CBP coactivator, which acts as an inhibitor of the IL-6 signaling pathway through a classic feedback mechanism. These findings further confirm that p21WAF1/CIP1/SDI1 is a converging point for the regulation of several intracellular signaling cascades.

Acknowledgments—We thank Dr. M. Roussel, Dr. X. Y. Yang, Dr. F. Gouilleux, and Dr. R. Potedar for the gift of various expression vectors.

REFERENCES

1. Hirano, T. (1998) Int. Rev. Immunol. 16, 249–284
2. Conover, J., Ip, N., Poseyminrou, W., Bates, B., Goldfarb, M., DeChiara, T., and Vazquezpolus, G. (1993) Development 119, 559–565
3. Penicca, D., Shaw, K., Swanson, T. A., Moore, M. W., Shelton, D. L., Zichok, K. A., Rosenthal, A., Taga, T., Paoni, N. F., and Wood, W. I. (1995) J. Biol. Chem. 270, 10915–10922
4. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
5. Ihle, J. (1995) Nature 377, 591–594
6. Hoey, T., and Schindler, U. (1998) Curr. Opin. Genet. & Dev. 8, 582–587
7. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mit
Functional Interaction of STAT3 Transcription Factor with the Cell Cycle Inhibitor p21 WAF1/CIP1/SDI1

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J. Biol. Chem. 2000, 275:18794-18800.
doi: 10.1074/jbc.M001601200 originally published online April 11, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001601200

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