Cyclin D1 Represses STAT3 Activation through a Cdk4-independent Mechanism*

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STAT3 transcription factors are cytoplasmic proteins that induce gene activation in response to cytokine receptor stimulation. Following tyrosine phosphorylation, STAT3 proteins dimerize, translocate into the nucleus, and activate specific target genes. Activation is transient, and down-regulation of STAT3 signaling occurs within a few hours. In this study, we show that cyclin D1 inhibits STAT3 activation. In co-immunoprecipitation and pull-down assays, cyclin D1 was found to associate with the activation domain of STAT3 upon interleukin-6 stimulation. Overexpression of cyclin D1 inhibited transcriptional activation by STAT3 proteins. This effect was not shared by cyclin E, was independent of association with Cdk4, and was unaffected by inhibitors of Cdk4. Whereas cyclin D1 had no effect on the steady-state level of STAT3 proteins in the cytoplasm, it was found to reduce the STAT3 nuclear level in HepG2 cells. These results suggest a model by which cyclin D1 is part of a feedback network controlling the down-regulation of STAT3 activity and highlight a new activity for this cell cycle regulatory protein.

The JAK/STAT pathways are activated by various growth factors and cytokines such as epidermal growth factor and interleukin-6 (IL-6). Binding of these cytokines to their receptors activates the JAK tyrosine kinases, followed by tyrosine phosphorylation of the receptors. This leads to activation and homo- or heterodimerization of the STAT1/3 transcription factors, translocation into the nucleus, and activation of target genes (1, 2). STAT3 activation is often associated with cell growth or transformation, and disruption of the STAT3 gene causes embryonic lethality around remove embryonic day 7.5, confirming a role for STAT3 in cell survival and proliferation in embryonic development (3). Recent experiments indicate that STAT3 transcription factors induce cell transformation and can be considered as oncogenes (4, 5). Tumor-derived cell lines or samples from human cancer contain very frequently activated forms of STAT3, and all src-transformed cell lines exhibit activated STAT3 (4, 6–9). Coexpression of a dominant-negative form of STAT3 is sufficient to block cell transformation by Src (7, 9). Inhibition of STAT3 transcriptional activity can decrease Bcl-xL expression and induces apoptosis in U266 cells (10) as well as in cultures of primary human myeloma cells (4). Finally, STAT3 is also required for the IL-6-mediated activation of the c-myc gene (11) and for the expression of cyclin D1 (5), two key components in the regulation of cell cycle progression from G1 to S phase.

One of the important questions to be resolved is what molecular basis governs the activation of cell cycle progression by STAT3 transcription factors and how these proteins are inactivated. Cell cycle activation is coordinated by D-type cyclins, which are rate-limiting and essential for the progression through the G1 phase of the cell cycle (12). D-type cyclins bind to and activate the cyclin-dependent kinases Cdk4 and Cdk6 (13). This binding allows for efficient phosphorylation of the critical downstream target of cyclin D, the retinoblastoma protein Rb. In the G1 phase of the cell cycle, Rb is hypophosphorylated and binds to the E2F family of cell cycle transcription factors (14). This Rb-E2F complex forms an active transcriptional repressor at the promoter of cell cycle genes that is responsible for growth suppression (15). The binding of Rb to E2F and the ability to repress transcription are modulated by the state of phosphorylation of Rb catalyzed by cyclin-dependent kinases. The complete phosphorylation of Rb by cyclin D1/Cdk4 and cyclin E/Cdk2 is necessary for its inactivation, resulting in the release of Rb-associated E2F and the activation of S phase genes (16–18).

In the past 3 years, several observations have raised the possibility that cyclin D1 has roles at other stages of the cell cycle and that these new activities are unrelated to its function as a Cdk regulatory subunit (19). Cyclin D1 can activate estrogen receptor transcription through a direct interaction with the ligand-binding domain of the receptor (20, 21). This effect is independent of Cdk4 and is explained by the recruitment of the SRC1a family of coactivators by cyclin D1 (22). In line with these observations, cyclin D1 inhibits transcriptional activation by the v-myb oncogene, and this repression is also independent of a complex formation with Cdk4 (23, 24). The transcriptional activity of another Myb-like protein, DMP1, is also antagonized by D-type cyclins through a Cdk-independent mechanism (25). Finally, cyclin D1 has been shown to repress muscle differentiation and MyoD-mediated transcription in part through an Rb-independent mechanism (26, 27). Taken together, these studies establish a new Cdk-independent role of cyclin D1 as a transcriptional regulator (19).

STAT proteins activate the expression of the cyclin D1 gene through the recognition of a conserved element in the promoter of the gene (5, 28). As one of the target genes activated by the STAT cascade, cyclin D1 is probably an important mediator of the STAT-dependent growth of hematopoietic cells. However, the exact role of this protein in this signaling pathway remains to be determined. In this study, we show that following IL-6...
stimulation, cyclin D1 interacts with STAT3 and inhibits its transcriptional activity. This effect is not shared by cyclin E, which is not inactivated by cyclin D1, and is also independent of Cdk4, and is related to reduced STAT3. Repression of STAT3 by Cyclin D1

MATERIALS AND METHODS

Cell Culture and Reagents—Cell lines obtained from the American Type Culture Collection (Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum or in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 ng/ml granulocyte/macrophage colony-stimulating factor for TF1 cells. Purified recombinant cytokines were obtained from Dr. K. Turner (Genetics Institute, Boston, MA). Vectors expressing cyclin D1 (pRC/cmv), p16INK4 (pcDNA3), and cyclin D1-KE (pRC/cmv) were kind gifts of Drs. P. Whaye and R. Bernards, respectively. The pGal-STAT3 and pGal-VP16 fusion proteins and the Gal4-luciferase reporter gene system were kindly provided by Drs. D. E. Levy and F. Gouilleux. Plasmid expressing gp130

has been described previously (29). Polyclonal antibodies recognizing STAT3 (C20) and cyclin D1 (H-295) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies directed against phospho-STAT3 Tyr705 and phospho-STAT3 Ser727 were obtained from New England Biolabs Inc. (Beverly, MA).

Plasmid Constructs—Expression vectors were used as templates for polymerase chain reaction amplification of various portions of cyclin D1 and STAT3. GST-cyclin D1 fusion constructs were prepared in pGEX-2T using oligonucleotide primers containing BamHI and EcoRI restriction sites corresponding to amino acids 1–295 of cyclin D1. Histidine-tagged STAT3 fusion constructs were prepared in pET15b using oligonucleotide primers containing BamHI restriction sites corresponding to amino acids 716–770 of STAT3.2

Transient Transfections and Preparation of Nuclear Extracts—Transfection experiments were done using the calcium phosphate precipitation method and were repeated at least five times. COS cells were transfected following the DEAE-dextran method. The amount of transcribed DNA was kept constant by addition of appropriate amounts of the parental empty expression vector (pRC/cmv). For nuclear extracts, HepG2 cells were plated in 15-cm plates at a density of 8 × 10^6 cells/plate and serum-starved for 1–3 days; and after two washings with cold phosphate-buffered saline, nuclear extracts were prepared according to the method of Lee et al. (30). Briefly, 1 ml of ice-cold extraction buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) was added to the cells. After three cycles of freeze-thawing, cytoplasmic extracts were recovered by centrifugation at 12,000 rpm for 5 min, and pellets were resuspended in buffer containing 20 mM Hepes (pH 7.9), 1.5 mM MgCl2, 420 mM KCl, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Following a 30-min incubation at 4 °C, nuclear extracts were spun down at 12,000 rpm for 5 min.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were preincubated for 5 min at room temperature in 25 mM NaCl, 10 mM Tris (pH 7.5), 1 mM MgCl2, 5 mM EDTA (pH 8), 5% glycerol, and 1 mM dithiothreitol with 1 μg of poly(dI-dC) as a non-specific competitor. Where indicated, 5-μg 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 (siem (9)–CATTTCCCGTAAATCTTGTCG–3) was end-labeled using T4 kinase, and 10 pg of probe (20,000 cpm) was then added to the protein mixture for 15 min. Samples were loaded on a 5% polyacrylamide gel (30:1) and separated by electrophoresis in 50 mM Tris, 0.58 mM glycine, and 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 immunoprecipitated with the indicated antibodies overnight at 4 °C on a rotator, followed by addition of 40 μl of protein A-Sepharose for 1 h at 4 °C. Cyclin D1 was immunoprecipitated in the presence of 1% Brij 97, whereas STAT3 proteins were recovered in the presence of 1% Nonidet P-40. Immunoprecipitates were washed three times with lysis buffer (0.1 M KHP04, (pH 7.8) and 0.1% Triton X-100), boiled for 5 min, and loaded on polyacrylamide gel. Following electrophoresis, gels were washed five times and incubated overnight at 4 °C in Tris-buffered saline (10 mM Tris (pH 8) and 150 mM NaCl) containing 6% bovine serum albumin. Blots were then incubated with the indicated antibodies in Tris-buffered saline containing 6% bovine serum albumin and 0.1% Tween, washed five times, and further incubated with antibodies conjugated to horseradish peroxidase. Proteins were visualized using the ECL system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

Fusion Protein Purification and Pull-down Experiments—Bacterial cultures (Escherichia coli strain BL21 or DH5) expressing fusion proteins were grown in LB medium and induced with 1 μM isopropyl-β-D-thiogalactopyranoside after the absorbance had reached 0.6. Following centrifugation, bacterial pellets were lysed by sonication in phosphate-buffered saline containing 1% Triton X-100 and 1 mM EDTA. Extracts were recovered by centrifugation at 12,000 rpm for 5 min, and fusion proteins were purified by affinity chromatography following the procedure provided by the suppliers. 100–200 μg of purified His-STAT3 and Gal4-STAT3 (p9) were bound to 200–200 μg of GST-cyclin D1 coupled to glutathione beads and incubated for 30 min at 4 °C in binding buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl. Beads were then washed three times with binding buffer and one time with 50 mM

2 Details of constructs are available on request.
Tris-HCl (pH 8), boiled for 5 min, and loaded on polyacrylamide gel for Western blot analysis. The C20 polyclonal antibody was used to detect His-STAT3<sup>D1–716</sup>, corresponding to the carboxyl-terminal part of STAT3.

Luciferase Assays—HepG2 cells were plated in 24-well plates 24 h prior to transfection at a density of 3 × 10<sup>4</sup> cells/well. Transfected cells were washed twice with ice-cold phosphate-buffered saline, and 150 μl of lysis buffer was added to the wells. Extracts were then used directly to measure luciferase activity by integrating total light emission over a 10-s period using a Packard Topcount scintillation counter. Luciferase activity was normalized based on protein concentrations.

RESULTS

In Vivo Interaction between Cyclin D1 and STAT3 Proteins—To determine whether cyclin D1 might be involved in the regulation of STAT3 activation, we first investigated whether it could bind specifically to STAT3 proteins. Following IL-6 stimulation, nuclear extracts were recovered from HepG2 hepatoma cells, and co-immunoprecipitations were performed alternatively with polyclonal antibodies directed against cyclin D1 (Fig. 1A, lanes 2), polyclonal antibodies directed against STAT3 (lane 4), or nonspecific antibodies (lanes 1 and 3). Proteins present in the immunoprecipitates were revealed by immunoblotting with the reciprocal antibodies. In both cases, cyclin D1 and STAT3 were found to co-immunoprecipitate (Fig. 1A, compare lanes 1 and 3 with lanes 2 and 4). Similar effects were also observed in a different cell line that expresses the IL-6 receptor transducing complex, the human erythroleukemia TF1 cell line (Fig. 1B). This interaction was dependent on the presence of IL-6 since a very weak interaction was detected between the two proteins when cells were serum-starved for 2 days. By contrast, cyclin D1 and STAT3 co-immunoprecipitated in response to IL-6 stimulation (Fig. 1C, lanes 1–4). Whether the phosphorylation has a direct effect on the interaction or is only related to an increased quantity of STAT3 in the nucleus remains to be determined. Importantly, these co-immunoprecipitations were carried out using nuclear extracts from non-transfected cells; therefore, the association between cyclin D1 and STAT3 does not require that these proteins be overexpressed. Altogether, these results indicate that STAT3 signaling proteins interact with cyclin D1.

Inhibition of STAT3 Transcriptional Activity by Cyclin D1—Recent results have shown that cyclin D1 can inhibit the transcriptional activity of the Myod, v-Myb, and DMP1 transcription factors (23–26). Having shown that cyclin D1 and STAT3 co-immunoprecipitated in response to IL-6 stimulation (Fig. 1C, lanes 1–4), whether the phosphorylation has a direct effect on the interaction or is only related to an increased quantity of STAT3 in the nucleus remains to be determined. Importantly, these co-immunoprecipitations were carried out using nuclear extracts from non-transfected cells; therefore, the association between cyclin D1 and STAT3 does not require that these proteins be overexpressed. Altogether, these results indicate that STAT3 signaling proteins interact with cyclin D1.
pressing STAT3 and cyclin D1. Following transfection, cells were serum-starved and stimulated with IL-6, and luciferase activity was measured after 15 h in cytoplasmic extracts. Inclusion of a STAT3 expression vector in the transfection mixture led to a 5-fold increase in expression following cell stimulation (Fig. 2A, bars 1 and 2). Activation by STAT3 was inhibited in the presence of a cyclin D1 expression vector (Fig. 2A, compare bars 2 and 3). Importantl, cyclin D1 was also able to block transcriptional activation in the absence of a STAT3 expression vector, suggesting that the endogenous protein is also inhibited (Fig. 2A, compare bars 4–6). The same effect was also observed in the presence of leukemia inhibitory factor (Fig. 2B, compare bars 2 and 3) and oncostatin M (Fig. 2C, compare bars 2 and 3), indicating that the cyclin D1 inhibitory effect is shared by cytokines using the STAT3 signaling pathway. The cyclin D1-mediated inhibitory effect on IL-6- and leukemia inhibitory factor-induced transactivation increased when increasing amounts of cyclin D1 expression vectors were included in the transfection mixture (Fig. 2D, compare bars 2–4 with bars 6–9). Thus, we concluded from these results that cyclin D1 can inhibit the transcriptional activity of STAT3 proteins.

Effect of Cyclin D1 on STAT3 DNA Binding in COS Cells—To explain the effect of cyclin D1 on STAT3 transcriptional activity, we first hypothesized that cyclin D1 might inhibit the DNA-binding activity of the transcription factor. To obtain a high transfection efficiency (>80%), COS-7 cells were transfected with vectors expressing the IL-6 receptor together with plasmids expressing STAT3 and cyclin D1. Under these experimental conditions, STAT3 DNA binding was induced following IL-6 stimulation (Fig. 3A, compare lanes 1 and 2). EMSA experiments were conducted in the presence of antibodies against STAT3 to prove that the binding was specific for this protein (Fig. 3A, compare lanes 4 and 5). Overexpression of cyclin D1 did not affect STAT3 DNA-binding activity (Fig. 3A, compare lanes 2 and 3), although cyclin D1 was clearly overexpressed (compare lanes 7 and 8 in the lower panel) and did interact with STAT3 (compare lanes 7 and 8 in the upper panel). In line with these results, Western blot experiments indicated that overexpression of cyclin D1 in COS cells did not affect the nuclear expression of STAT3 DNA binding in COS cells. However, this conclusion depends on the stoichiometry, and it remains to be determined if all STAT3 proteins are in complex with cyclin D and whether STAT3 is capable of binding both cyclin D1 and DNA simultaneously.

The Inhibitory Effect of Cyclin D1 Is Mediated through Its Interaction with the Activation Domain of STAT3—Following the study of the effect of cyclin D1 on STAT3 DNA binding, we then hypothesized that it should inhibit the activity of a chimeric Gal4-STAT3 fusion protein. Using a Gal4-dependent luciferase reporter gene, we found that cyclin D1 was able to repress transactivation by Gal4-STAT3 (Fig. 4, compare bars 2 and 3). Importantly, cyclin D1 had no effect on the transcriptional activity of a control Gal4-VP16 fusion protein (Fig. 4, compare bars 5 and 6). This result further suggested that the effect of cyclin D1 was probably not mediated through inhibition of DNA binding and also indicated that cyclin D1 may interact with the carboxyl-terminal activation domain of STAT3. To verify this, in vitro pull-down experiments were performed using bacterially produced full-length GST-cyclin D1 protein and His<sub>6</sub>-tagged STAT3 containing amino acids.
716–770 corresponding to the activation domain of STAT3 (STAT3Δ1–716) (Fig. 5A). We found that His-tagged STAT3Δ1–716 could be retained by a GST-cyclin D1 fusion protein immobilized on glutathione beads, whereas it did not bind to GST beads alone (Fig. 5B, compare lanes 1 and 2). To confirm this result, we also determined that GST-cyclin D1 could be retained by His-tagged STAT3Δ1–716 immobilized on beads, whereas it bound weakly to histidine beads alone (Fig. 5B, compare lanes 3 and 4). Pull-down experiments were also performed using HepG2 nuclear extracts mixed with immobilized GST or GST-cyclin D1. The results indicated that the endogenous STAT3 protein could also interact with GST-cyclin D1 (Fig. 5B, compare lanes 5 and 6).

Thus, we concluded from these results that cyclin D1 binds at least to the carboxyl-terminal activation domain of STAT3. Moreover, these pull-down experiments also suggest that the interaction between cyclin D1 and STAT3 is probably direct, although we cannot rule out the possibility that cyclin D1 functions via another partner that could be copurified from the bacteria with His-tagged STAT3 or GST-cyclin D1.

Cyclin D1 Inhibits STAT3-mediated Transactivation Independently of Its Cdk4 Partner—We then verified the specificity of the cyclin D1 inhibitory effects among the other cyclins. STAT3 activity is down-regulated in HepG2 cells during the first 6 h of IL-6 stimulation (31). To ascertain the physiological significance of the results, we reasoned that any cyclin that would affect the activity of STAT3 proteins should be expressed when the transcription factor is present in the nucleus. Among the different cyclins, cyclin A is induced at the beginning of the S phase of the cell cycle, whereas cyclin B appears at the end of the S phase and in G2. Therefore, these proteins are unlikely to modulate STAT3 activity. By contrast, cyclin D1 is induced in early G1, and cyclin E could be expressed in the middle of G1 (12). Therefore, cyclin E could theoretically also affect the transcriptional activity of STAT3. To verify this, HepG2 cells were cotransfected with a luciferase reporter construct together with plasmids expressing STAT3 and cyclin D1 or cyclin E. Transcriptional activation by STAT3 was almost completely abolished in the presence of a cyclin D1 expression vector, whereas

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**Fig. 4.** Cyclin D1 inhibits the transcriptional activity of a Gal4-STAT3 fusion protein. HepG2 cells were cotransfected with vectors expressing a Gal4-luciferase reporter gene (500 ng) together with vectors expressing a Gal4 fusion protein linked to the STAT3 activation domain (500 ng) in the presence (bar 3) or absence (bars 1 and 2) of a plasmid encoding cyclin D1 (CD; 10 ng). The same experiments were performed in parallel using a Gal4-VP16 plasmid (500 ng) (bars 4–6). Following transfection, cells were serum-starved for 15 h and stimulated overnight with IL-6 (10 ng/ml). Cytoplasmic extracts were then prepared and processed to measure luciferase activity. The mean of four transfections is shown.

**Fig. 5.** Cyclin D1 interacts with the activation domain of STAT3. A, shown is a representation of the carboxyl-terminal STAT3Δ1–716 fusion protein used in the pull-down experiments. B, His-tagged STAT3 fusion proteins corresponding to the activation domain of STAT3 (STAT3Δ1–716; 100–200 ng) were tested for binding to GST or to full-length GST-cyclin D1 (100–200 ng) immobilized on Sepharose beads (lanes 1 and 2). Samples were then washed four times and separated on 6% polyacrylamide gels, and STAT3 binding was detected by Western blotting using anti-STAT3 polyclonal antibodies (C20). Purified GST-cyclin D1 proteins (20 ng) were incubated for 30 min at 4 °C with histidine or His-tagged STAT3 immobilized on nickel-agarose beads (20 ng) (lane 3 and 4). Samples were then washed four times and separated on 6% polyacrylamide gels, and cyclin D1 binding was detected by Western blotting using anti-cyclin D1 polyclonal antibodies. In parallel, HepG2 cells were serum-starved for 48 h and then stimulated with IL-6 (10 ng/ml) for 2 h. Nuclear cell extracts (10–50 μg) were incubated for 30 min at 4 °C with GST or GST-cyclin D1 immobilized on Sepharose beads (lanes 5 and 6). STAT3 binding was detected by Western blotting using anti-STAT3 polyclonal antibodies.
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A.

HepG2 cells were cotransfected as described in the legend to Fig. 2 together with vectors expressing STAT3 alone (lanes 1 and 2) or with cyclin D1 (CD; lane 3) or cyclin E (CE; lane 4). Following transfection, cells were serum-starved for 15 h and stimulated overnight with IL-6 (10 ng/ml) (lanes 2–4). Cytoplasmic extracts were then prepared and analyzed by EMSAs using radiolabeled oligonucleotides encoding a STAT3 consensus binding site.

B.

Luciferase activity was evaluated as described for A. The mean of eight transfections is shown. In parallel, COS cells transfection of cyclin D1 (Fig. 6B, compare lanes 5 and 7). As previously reported for the v-Myb and DMP1 transcription factors (24, 25), the inhibition observed with the cyclin D1-KE mutant was consistently greater than that with the wild-type cyclin D1 (Fig. 6B, compare bars 3 and 4). It is conceivable that competition for cyclin D1 binding by endogenous Cdk4 might limit the inhibitory effect of cyclin D1 and its interaction with STAT3. If so, this would explain why the cyclin D1-KE mutant is a more potent STAT3 inhibitor than wild-type cyclin D1.

C.

To confirm these results, we also used a specific Cdk4 inhibitor, p16INK4. INK4 can compete with cyclin D1 for binding to Cdk4, but can also inhibit Cdk4 without dissociating cyclin D1, resulting in INK4-cyclin D-Cdk4 complexes (33). Significantly, cotransfection of p16INK4 had no effect on the cyclin D1-mediated inhibition of STAT3 transcriptional activity (Fig. 6C, compare bars 3 and 4), further suggesting that the inhibitory effect is mediated by “free” cyclin D1. Taken together, these results suggest that cyclin D1-mediated inhibition of the transcriptional activity of STAT3 is independent of the ability of cyclin D1 to activate Cdk4.

Cyclin D1 Reduces STAT3 Nuclear Levels—As a first attempt to characterize the inhibitory effect of cyclin D1 on STAT3 transcriptional activity in HepG2 cells, we hypothesized that cyclin D1 may also regulate the nuclear level of STAT3 in this cell line. To test this hypothesis, HepG2 cells were stably transfected with an expression vector encoding cyclin D1 or the corresponding parental plasmid. Following G418 selection, cyclin D1 was clearly overexpressed compared with control clones (Fig. 7A, lanes 1 and 2), STAT3 proteins, once activated in response to IL-6, become phosphorylated on Tyr705, dimerize, and translocate to the nucleus. To verify if the down-regulation of STAT3 transcriptional activity could be related to reduced nuclear expression, Western blot experiments were performed on cytoplasmic and nuclear extracts. Following IL-6 stimulation, cytoplasmic STAT3 was expressed (Fig. 7B, upper panel) and phosphorylated on Tyr705 in control cells as well as in two different clones overexpressing cyclin D1 (Fig. 7C, compare lanes 1–3 with lanes 4–9). However, the cytoplasmic phosphorylation of STAT3 on Tyr705 was reduced in clone 1 (Fig. 7C, compare lanes 2 and 3 with lanes 5 and 6). In the presence of cyclin D1, the expression level of STAT3 in the nucleus was very weak (Fig. 7B, lower panels, compare lanes 1–3 with lanes 4–9), but remained normal under control conditions. Interestingly, STAT3 was almost absent from the nucleus of clone 2, and these cells were found to express higher levels of cyclin D1 (data not shown). Importantly, this effect was not due to a general defect of STAT3 translocation since nuclear expression of the transcription factor could be detected upon a longer exposure of the blot (Fig. 7D, compare lanes 1–4 with lane 5).

We concluded from these results that cyclin D1 decreased the steady-state level of STAT3 proteins in the nucleus. Thus, this down-regulation might explain the negative effect of cyclin D1 on STAT3 transcriptional activity in HepG2 cells. If this hypothesis is correct, we then reasoned that cyclin D1 should not interact with STAT3 at the time of its maximal activation, whereas STAT3 should disappear from the nucleus with the appearance of cyclin D1-STAT3 complexes. To verify this, HepG2 cells were stimulated with IL-6, and nuclear activation of STAT3 was evaluated in parallel with the appearance of cyclin D1-STAT3 complexes. After 15 min, STAT3 translocated.

cyclin E was unable to affect STAT3 proteins, confirming the specificity of cyclin D1 inhibition (Fig. 6A, compare bars 2–4). To extend these results, we then investigated if Cdk4, the kinase partner of cyclin D1, was required in this inhibitory effect. To this end, we used a mutant of cyclin D1 (cyclin D1-KE) that carries a mutation in the cyclin box changing the lysine at amino acid 114 to glutamic acid. This alteration resulted in a mutant cyclin D1-KE protein that fails to bind to Cdk4 (32). Like wild-type cyclin D1, the cyclin D1-KE mutant inhibited transcriptional activation by STAT3 (Fig. 6B, bar 4). Importantly, EMSA experiments indicated that overexpression of cyclin D1-KE might not affect STAT3 DNA-binding activity in COS cells, confirming the results obtained with wild-type cyclin D1 (Fig. 6B, compare lanes 5 and 7). As previously reported for the v-Myb and DMP1 transcription factors (24, 25), the inhibition observed with the cyclin D1-KE mutant was consistently greater than that with the wild-type cyclin D1 (Fig. 6B, compare bars 3 and 4). It is conceivable that competition for cyclin D1 binding by endogenous Cdk4 might limit the inhibitory effect of cyclin D1 and its interaction with STAT3. If so, this would explain why the cyclin D1-KE mutant is a more potent STAT3 inhibitor than wild-type cyclin D1.
to the nucleus and was phosphorylated on Tyr705 and Ser727; however, no interaction could be detected between STAT3 and cyclin D1 in co-immunoprecipitation experiments (Fig. 7E, lanes 1 and 2). Interestingly, cyclin D1 and STAT3 were found to interact after 2 h of stimulation, when the nuclear level of STAT3 and its phosphorylation status were found to decrease (Fig. 7E, lanes 2 and 3). These results, together with the evidence regarding the interaction of STAT3 and cyclin D1, led us to propose that the transcriptional inhibition of STAT3 by cyclin D1 could be mediated through reduced nuclear expression in HepG2 cells.

DISCUSSION

The results presented in this study describe a new pathway for inhibiting STAT3 activation and establish a new role for cyclin D1 as a STAT3 inhibitor. We have shown that cyclin D1 can interact with STAT3 proteins and block their activity, suggesting a novel biological role for cyclin D1 in the feedback regulation of IL-6-type cytokine signaling pathways.

At the nuclear level, STAT3 activation is transient, and activated transcription factors disappear from the nucleus within 1–6 h after ligand stimulation (34). 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 (35). Phosphorylated STAT proteins can also be detected in association with ubiquitin, and proteasome inhibitors prolong the activation of these transcription factors, suggesting that proteolytic degradation participates in the negative regulation of nuclear STAT activation (36). Association with inhibitory molecules has also been recently described: the PIAS family proteins are negative regulators of STAT activity, and they associate with STAT proteins and inhibit DNA binding (37). Additional stimuli can also block the STAT signaling cascade in the nucleus, and we have recently shown that the cell cycle inhibitor p21WAF1/CIP1/SDI1 also blocks the IL-6 signaling pathway through a feedback mechanism (31). Thus, multiple levels of regulation act upstream or at the level of STAT3 proteins, and the results presented in this study extend the observation that gene activation by STAT3 proteins is affected by signals that control cell cycle progression.

Cell cycle progression is activated by the binding of D-type cyclins to Cdk4 and Cdk6; therefore, cyclin D1 is generally
considered a Cdk activator (13). However, the cyclin D1-mediated repression of STAT3 transcriptional activity is unrelated to its function as a Cdk4 regulatory subunit, whereas cyclin E is unable to affect STAT3 proteins. Therefore, the transcriptional activity of cyclin D1 is probably unique among the cyclins and is not mediated by Cdk4. Importantly, similar observations have been reported for the estrogen-, v-Myb-, DMP1-, and MyoD-regulated genes, where cyclin D1 regulates the transcriptional activity independently of Cdk4 (23–26). Taken together, these observations indicate that cyclin D1 can act via two different mechanisms: as a Cdk activator, it regulates cell cycle progression; and as a transcriptional regulator, it modulates the activity of transcription factors (19). This effect remains to be clarified, however, since cyclin D1 is a positive regulator of estrogen receptors, whereas it inhibits the activity of the v-Myb, DMP1, MyoD, and STAT3 transcription factors.

A few possibilities can be raised concerning the molecular mechanisms whereby STAT3 is inhibited by cyclin D1. The cyclin is able to inhibit the transcriptional activity of a chimeric Gal4-STAT3 protein containing the activation domain of STAT3. The domain that modulates the interaction between cyclin D1 and STAT3 proteins remains to be fully identified; but, as a first attempt to map this region, we have shown that cyclin D1 is at least able to interact with the activation domain of STAT3. In light of this result, we might speculate that cyclin D1 could occlude this domain, thereby blocking the interactions of STAT3 with the RNA polymerase II transcriptional machinery or with its essential cofactors such as CBP/p300 (38). Steric hindrance would, in this case, lead to transcriptional repression. Interestingly, CBP/p300 has been recently shown to bind the carboxyl-terminal part of STAT3 (38), and cyclin D1 has been shown to interact with essential transcriptional coactivators (22). Additionally, the binding of cyclin D1 within the activation domain of STAT3 could inhibit the phosphorylation of serine 727, a critical residue involved in the transcriptional activity of STAT3 (39). Again, interaction of cyclin D1 with STAT3 could reduce the accessibility to Ser727.

A second hypothesis can probably be raised concerning the molecular mechanisms whereby STAT3 signaling is inhibited, as we found that cyclin D1 decreased the steady-state level of STAT3 proteins in the nuclei of HepG2 cells. This was not a general defect since STAT3 can enter the nucleus in the presence of cyclin D1, but to a lesser extent. These observations raise the possibility that STAT3 nuclear localization is regulated following its association with cyclin D1, maybe in a cell-cycle dependent manner. In line with this hypothesis, the transcriptional activity of STAT1 has also been shown to be differentially regulated during cell cycle progression (40). This effect remains to be clarified, but we speculate that STAT3 inhibition is mediated through two potential mechanisms: occlusion of the transactivation function and a decrease in nuclear amount.

Altogether, these results point to a novel biological role for cyclin D1 as an important inhibitor of STAT3 transcriptional activity. We propose a model by which IL-6 stimulation induces the interaction between cyclin D1 and STAT3 proteins, leading to the feedback down-regulation of STAT3 activation. These findings extend the role of cyclin D1 as a converging point of several intracellular signaling cascades.

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