Cyclin D1 Represses p300 Transactivation through a Cyclin-dependent Kinase-independent Mechanism

Received for publication, March 23, 2005, and in revised form, June 9, 2005
Published, JBC Papers in Press, June 10, 2005, DOI 10.1074/jbc.M503188200

Maofu Fu‡, Chenguang Wang‡, Mahadev Rao‡, Xiaofang Wu‡, Toula Bouras‡, Xueming Zhang‡, Zhiping Li§, Xuanmao Jiao§, Jianguo Yang§, Anping Li‡, Neil D. Perkins§, Bayar Thimmmapaya§, Andrew L. Kung†, Alberto Munoz,**, Antonio Giordano‡‡, Michael P. Lisanti§§, and Richard G. Pestell†††

From the ‡Lombardi Comprehensive Cancer Center, Department of Oncology, Georgetown University, Washington, D. C. 20057, §Division of Gene Regulation and Expression, School of Life Sciences, University of Dundee, MSI/WTB Complex, Dow Street, Dundee DD1 5EH, Scotland, United Kingdom, †Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611, ‡Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, **Instituto de Investigaciones Biomedicas Alberto Sols, Consejo Superior de Investigaciones Cientificas-Universidad Autonoma de Madrid, Arturo Duperier 4, E-28029 Madrid, Spain, ‡‡Department of Pathology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, and §§Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

Cyclin D1 encodes a regulatory subunit, which with its cyclin-dependent kinase (Cdk)-binding partner forms a holoenzyme that phosphorylates and inactivates the retinoblastoma protein. In addition to its Cdk binding-dependent functions, cyclin D1 regulates cellular differentiation in part by modifying several transcription factors and nuclear receptors. The molecular mechanism through which cyclin D1 regulates the function of transcription factors involved in cellular differentiation remains to be clarified. The histone acetyltransferase protein p300 is a co-integrator required for regulation of multiple transcription factors. Here we show that cyclin D1 physically interacts with p300 and represses p300 transactivation. We demonstrated further that the interaction of the two proteins occurs at the peroxisome proliferator-activated receptor γ-responsive element of the lipoprotein lipase promoter in the context of the local chromatin structure. We have mapped the domains in p300 and cyclin D1 involved in this interaction. The bromo domain and cysteine- and histidine-rich domains of p300 were required for repression by cyclin D1. Cyclin D1 repression of p300 was independent of the Cdk and retinoblastoma protein-binding domains of cyclin D1. Cyclin D1 inhibits histone acetyltransferase activity of p300 in vitro. Microarray analysis identified a signature of genes repressed by cyclin D1 and induced by p300 that promotes cellular differentiation and induces cell cycle arrest. Together, our results suggest that cyclin D1 plays an important role in cellular proliferation and differentiation through regulation of p300.

The cyclins and the associated cyclin-dependent kinases (Cdks) govern proliferation of mammalian cells. The regulation of cyclin subunit cyclin binds and activates their catalytic partners, or Cdks, allowing phosphorylation of a series of critical cellular substrates, thereby promoting cell cycle progression (1). D-type cyclins fluctuate in abundance during cell cycle progression, induced by mitogenic stimulation, and in the case of cyclin D1 serve as a key target in oncogenic and mitogenic signaling. Phosphorylation of pRb in normal cells by cyclin D/Cdk4/6 is thought to induce structural changes of pRb and in turn allow sequential phosphorylation by cyclin E/Cdk2 and cyclin A/Cdk2 (2, 3). Cyclin D-Cdk complexes associate with several other proteins, including cell cycle inhibitors of the p27KIP1 and p21CIP1 family to regulate the functional activity of these inhibitors in trans (1). Clinical observations have identified cyclin D1 overexpression as a frequent occurrence in human breast tumors, lymphomas, and several other tumor types. Molecular genetic analysis of cyclin D1 function in the mouse demonstrates an essential role for cyclin D1 in normal mammary gland development and nonredundant functions for the D-type cyclins in hematopoietic stem cell expansion (4). The terminal alveolar breast bud developmental defect in cyclin D1-deficient mice was recapitulated by deficiency of either I KKα, mutation of NIK (Aly) (5), deletion of the gene for osteoprotegerin (Opg), or its ligand (OPGL also known as RANKL) (6, 7). Although substantial redundancy exists among the D-type cyclins for cellular proliferation, analysis of cyclin D1−/− animals identified an essential role for cyclin D1 in cellular migration, cellular survival, angiogenesis, and adipocyte differentiation (8–10). However, the mechanisms by which cyclin D1 regulates such diverse functions are not fully understood.

Several recent studies have identified functional interactions between cyclin D1 and diverse transcription factors. Cyclin D1 inhibits the activity of more than 30 distinct transcription factors (8). Mutational analysis demonstrated that the regulation of transcription factor activity by cyclin D1 was independent of the residues required for binding Cdk (10, 11). Cyclin D1-deficient animals show enhanced adipocyte differentiation in response to PPARγ ligands. As this phenotype was reversed...
Repression of p300 Transactivation by Cyclin D1

by reintroduction of cyclin D1, the repression of PPARγ by cyclin D1 has been considered an important physiological function. Cyclin D1 inhibits PPARγ transcriptional activity independently of the Cdk-binding domain of cyclin D1 (10, 11). In addition to cyclin D1, the transcriptional co-activator p300 plays an essential role in PPARγ function (12).

The p300/CBP orthologs encode proteins that coordinate transcription factor function through distinct subdomains (13). The domains conserved between p300 and CBP include a histone acetyltransferase (HAT) domain, a bromo domain, three cysteine- and histidine-rich domains (CH), and a cell cycle regulatory domain (CRD1). p300 was initially cloned as an E1A interacting protein (14-16), and the formation of the multimeric complex between p300 and E1A is important in overcoming normal growth control, as E1A mutants that fail to bind p300 cannot efficiently transform cells (17). p300 also forms a physical bridge between transcription factors and the basal transcription apparatus to coordinate regulation of gene transcription. The subdomains of p300 that encode HAT function alter the acetylation of lysine residues on histones, thereby altering accessibility of transcription factors to target gene promoters. The bromo domain of p300 recognizes and facilitates binding of p300 to acetylated lysine residues of histones and transcription factors such as p53 (18). Direct acetylation of transcription factors by p300 also alters transcription factor activity (19, 20).

The ability of p300 to augment the activity of transcription factors involved in both cell proliferation and differentiation suggests mechanisms must exist to coordinate p300 functions with the cell cycle machinery. p21CIP1/WAF1 regulates p300 and p300 also alters transcription factor activity (19, 20). The bromo domain of p300 recognizes and facilitates binding of p300 to acetylated lysine residues of histones and transcription factors such as p53 (18). Direct acetylation of transcription factors by p300 also alters transcription factor activity (19, 20). The ability of p300 to augment the activity of transcription factors involved in both cell proliferation and differentiation suggests mechanisms must exist to coordinate p300 functions and thereby coordinate growth and differentiation signals or functionally inactivate the putative tumor suppressor function of p300 during tumor progression (25).

Here studies were conducted to examine the mechanisms underlying the physiologically relevant role of cyclin D1 as an inhibitor of PPARγ function and adipocyte differentiation (10). As p300 is a limiting factor in PPARγ function, we assessed the role of cyclin D1 in regulating p300 function. We show that cyclin D1 physically interacts with p300 and represses p300 transactivation independent of its Cdk- and pRb-binding domains. Microarray analysis identified a signature of genes that are repressed by cyclin D1 and induced by p300. Collectively, our results suggest that cyclin D1 plays an important role to modulate p300 activity and its target gene expression.

EXPERIMENTAL PROCEDURES

Reagents, Reporter Genes, Expression Vectors, DNA Transfection, and Luciferase Assays—The (AOX), LUC (acyl-coenzyme A oxidase triple PPARγ-responsive element (PPARE) luciferase) reporter gene, pCMX-PPARE, Gal4-π300 plasmids (24, 33), and the UAS, E1B, TATA-LUC were described previously (34). The Dkk1-LUC reporter (4-2400 Dkk1-LUC (35), the FKHR-responsive gene reporter, 2XFHRE-LUC (36), the interferon-responsive element GAL4-LUC (37), and the luciferase reporter plasmid containing 3850 to +45 bp of the TFSP1 (mouse thrombospondin 1) promoter (38) were described elsewhere. E. coli p300 constructs were a kind gift from Dr. Bayar Thimmapaya (2, 16, 40). The p21/CIP1/WAF1 LUC DNA, a gift from Dr. W. El Deiry, was cloned into the expression vector pCMV5. Cyclin D1 mutants were generated by PCR and subcloned into p3XFLAG-CMV-10 (Sigma) (10). The p300- and p300 mouse embryonic fibroblasts (MEFs), cyclin D1, and cyclin D1′/MEFs, and 3T3 cells (cyclin D1′/MEFs, and cyclin D1′/MEFs) were described previously (41, 42). Cells were transfected by Superfect Transfection reagent (Qiagen, Valencia, CA) as described elsewhere (43). The medium was changed after 5 h; cells were treated with ligand or vehicle as indicated in the figure legends, and luciferase activity was determined after 24 h. Luciferase activity was normalized for transfection efficiency with β-galactosidase or Renilla reporters as an internal control. Luciferase assays were performed using a fluoroscan microplate reader at an excitation wavelength of 360 nm (Vantage SE, BD Biosciences) for GFP-positive cells. MEFS were infected with either MSCV-IRESGFP vector or MSCV-cyclin D1-IRESGFP and were used to probe Affymetrix U74Av2 arrays (Affymetrix, Santa Clara, CA). Probe synthesis and hybridization were performed according to the manufacturer’s manual (see eukaryotic target preparation section at www.affymetrix.com/support for details). Single array analysis and comparison analysis were performed after scanning the genechips, and all chips were scaled by setting the “Target Signal” at 5000 under “Affymetrix Weight” and normalized to the median. The data were then transferred to the data base. Two sets of arrays (either p300+/− or p300+/− or cyclin D1 vector control), three samples within each group, were compared, and nine comparison analyses were generated. The genes with change calls consistently increased or decreased were selected using the Affymetrix Data Mining Tools and were further filtered based on absolute change using the t test and detection cells. To visualize expression difference of the selected genes, color coding was performed using Matlab software. Immunoprecipitation and Western Blot—293T cells were transfected with expression vectors for cyclin D1 and p300. Thirty hours after transfection, the cells lysates were prepared in 600 μl of cell lysis buffer (10 mM HEPES, pH 7.5, 150 mM KC1, 0.4 mM EDTA, 10 mM sodium fluoride, 0.2% Nonidet P-40, 1 ml dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride with proteinase inhibitors (Roche Diagnostics, cat.no.1169355)). 600 μg of cellular lysate was subjected to immunoprecipitation with 10 μl of anti-Cyclin D1 or cyclin D1 antibodies (Santa Cruz Biotechnology) and 30 μl of protein A-agarose beads at 4 °C overnight. Normal rabbit IgG was used as a negative control. The beads were washed with 800 μl of cell lysis buffer five times, resuspended in 30 μl of cell lysis buffer plus 6 μl of SDS-PAGE loading buffer, and denatured by heating at 95 °C for 5 min. Proteins were dissolved in 10% SDS-PAGE. The membrane was blotted with either anti-p300 or anti-cyclin D1 antibody (DCS-6, Santa Cruz Biotechnology) at room temperature for 1 h and then washed three times with 0.05% Tween 20/phosphate-buffered saline. The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. The immunoreactive proteins were visualized by an enhanced chemiluminescence system (Amersham Biosciences).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP analysis was performed as described previously (45). 2 × 107 cyclin D1+/− or cyclin D1′-/− MEF cells were grown in Dulbecco’s modified Eagle’s medium with 10% charcoal-dextran-stripped serum for 3 days. Upon treatment, the cells were cross-linked by adding 1.0% formaldehyde buffer contain-
Repression of p300 Transactivation by Cyclin D1

RESULTS

Cyclin D1 Represses p300 Transactivation—Previously, we have shown that cyclin D1 inhibits PPARγ function and adipocyte differentiation (10, 11). p300 was shown previously to increase both basal and ligand-induced PPARγ-dependent activity (10). PPARγ ligand enhances p300 recruitment to PPARγ (51). As p300 is indispensable for the full activation of PPARγ and adipocyte differentiation (12), we examined the possibility that cyclin D1 could inhibit PPARγ function through repression of p300. The activity of the PPARγ-responsive reporter gene (AOX)LUC was assessed in 293 cells. In the presence of ligand (rosiglitazone), PPARγ expression enhanced (AOX)LUC activity 9–10-fold (Fig. 1A). p300 enhanced (AOX)LUC activity 9-fold and in the presence of ligand increased the reporter activity 15-fold. Cyclin D1 expression reduced p300-mediated PPARγ basal and ligand-induced activity by 30–40% (Fig. 1A). These results suggest PPARγ activity is determined in part by the relative abundance of p300 and cyclin D1 in the cells.

A broad array of prior studies has demonstrated that p300 activates transcription when fused to the Gal4 or E2 DNA-binding domains (48, 52–56). To examine the role of cyclin D1 in regulating p300 transactivation function, the activity of p300 linked to a Gal4-DNA-binding domain (Gal4-DDB) was assessed by using a heterologous reporter system that encodes multimeric Gal4-DNA-binding sites linked to a luciferase reporter gene (UAS2,E1B,TATA,LUC) as described previously (22, 33). We next determined whether cyclin D1 was sufficient for repression of p300. Co-expression of cyclin D1 repressed Gal4-p300 activity ~3-fold compared with equal amounts of empty expression vector (Fig. 1B). A detailed dose response confirmed that cyclin D1 repressed p300 activity at low molar ratios, obviating concerns of spurious plasmid effects (57) (Fig. 1, C and D). These results contrast with our previous findings that cyclin D1 enhances activity of Gal4-ERα (58) and that cyclin D1 does not affect the transactivation function of Gal4-VP16 (59).

The activity of p300 was compared in randomly cycling cyclin D1+/− and cyclin D1−/− cells with activity normalized to Renilla luciferase activity. The basal activity of UAS2,E1B,TATA,LUC was lower in cyclin D1−/− cells compared with cyclin D1 wild-type (Fig. 1E, lane 1 versus 2). p300 activity was increased 15-fold compared with the Gal4-DDB in the cyclin D1 wild-type cells (Fig. 1E, lane 1 versus 3). p300 activity was induced some 60-fold in the cyclin D1−/− cells (Fig. 1E, lane 2 versus 4). We next examined the role of cyclin D1 in attenuating serum-induced p300 activity. The addition of 10% serum induced Gal4-p300 activity 25-fold in the cyclin D1−/− cells (Fig. 1F). As the cyclin D1−/− cells show reduced cellular proliferation, and cells were treated with serum for 12 h, the increased activity of Gal4-p300 was not due to an alteration in cellular number during the time of transfection. These studies suggested that the loss of cyclin D1 enhanced p300 activity and indicated that cyclin D1 inhibition of p300 activity is selective.

In view of the finding that expression of cyclin D1 inhibited activity of p300 transactivation, we examined the possibility that cyclin D1 may physically associate with p300. Immunoprecipitation Western blotting was conducted of 293T cells transfected with expression vectors for cyclin D1 and p300. Immunoprecipitation of cyclin D1 and subsequent Western blotting demonstrated the presence of both cyclin D1 and p300 in the immunoprecipitate (Fig. 2A, lane 3). The reciprocal immunoprecipitation analysis using antibody to p300 demonstrated a co-precipitation of p300 and cyclin D1 (Fig. 2A, lane 5). Together these studies demonstrate cyclin D1 is associated with p300 in cultured cells. To determine whether cyclin D1 and p300 co-associated in the context of the local chromatin structure of a PPARγ target gene repressed by cyclin D1, ChIP assays were conducted. Cyclin D1 is known to inhibit ligand-induced activity of the synthetic PPARγ. We therefore examined the protein complexes recruited to the PPARγ of the endogenous murine LPL promoter. A comparison was made between cyclin D1+/− and cyclin D1−/− MEFs. Cyclin D1 was reintroduced into cyclin D1−/− MEFs by transfecting with either the MSCV-cyclin D1-IRESGFP or the MSCV-IRESGFP control virus. Cells were treated with either differentiation medium or troglitazone (5 μM) compared with vehicle. The presence of cyclin D1 introduced into cyclin D1−/− cells was identified by the FLAG epitope used in ChIP assays (Fig. 2B). The addition of differentiation medium reduced the abundance of p300 at the PPARγ of the LPL promoter in cyclin D1−/− cells. Co-expression of cyclin D1, as identified by the presence of the FLAG tag, was associated with the presence of p300 (Fig. 2B, lane 1 versus 3 and lane 2 versus 4). These findings are consistent with the model in which cyclin D1 and p300 are co-localized to the promoter sequences of target genes repressed by cyclin D1 and regulated by p300.

Cyclin D1 Repression of p300 Activity through Recruitment of HDAC—Transcriptional co-repression involves several distinct multiprotein complexes, including members of either the NcoR/mSin3/VHAD family or the NuRD family (60–62). To determine whether the repression of p300 by cyclin D1 involved proteins with histone deacetylase (HDAC) activity, 293 cells
and cyclin D1\(^{+/+}\) and cyclin D1\(^{--}\) cells transfected with Gal4-p300 were assessed. p300 activity was inhibited by co-expression of cyclin D1 in 293 cells, and the addition of TSA reduced this inhibition 80% \((p < 0.05)\) (Fig. 3A). These studies suggested both HDAC-dependent and HDAC-independent functions regulated cyclin D1-dependent inhibition of p300 activity. Although expression of HDAC alone did not inhibit p300 activity in 293 cells (Fig. 3B), co-expression of HDAC1 with cyclin D1 further enhanced cyclin D1-dependent repression of p300 (Fig. 3B). The activity of Gal4-p300 was 6–7-fold lower in cyclin
D1−/− cells when normalized to the internal control of Renilla luciferase activity (Fig. 3C). The addition of TSA induced Gal4-p300 ~2-fold in cyclin D1−/− cells but augmented Gal4-p300 activity 10–12-fold in cyclin D1+/− cells (Fig. 3D). These studies suggested that cyclin D1 repression of Gal4-p300 may involve the recruitment of proteins containing histone deacetylase activity. This finding is consistent with our previous finding that cyclin D1 inhibits PPARγ-mediated adipocyte differentiation through recruitment of HDACs (11).

Cyclin D1 Repression of p300 Activity Involves the Bromo and CH Domains of p300—To identify the regions of p300 required for repression by cyclin D1, a series of p300 mutant constructs was assessed (Fig. 4A). Recent studies confirmed expression of the Gal4-p300 expression vectors (53). For ease of comparison, the repression of p300 wild-type activity by cyclin D1 was normalized to 100%. The Gal4-p300 constructs, Δ61–1736 or Δ243–1736, reduced repression by 70–80%, suggesting the regions required for cyclin D1 repression lie between 242 and 1737. The Gal4-p300 construct 964–1922 was repressed 60%, and the construct 1–596 conveyed wild-type repression (Fig. 4B). Consistent with the previous studies, amino acids 1004–1044 were the site of repression by p21CIP1/WAF1 (Fig. 5, C and D). In keeping with previous studies, amino acids 1004–1044 were the site of repression by p21CIP1/WAF1 (Fig. 5, B and C) (24). p300 activity was repressed by cyclin D1; however, deletion of the CRD1 domain did not reduce the fold repression by cyclin D1 (Fig. 5, D and E). These studies suggest cyclin D1 does not repress p300 through the same domain affected by p21CIP1/WAF1.

Cyclin D1 Repression of p300 Is Independent of Cdk Binding—In order to examine the mechanisms by which cyclin D1 inhibits p300 activity, a series of cyclin D1 mutant constructs was assessed, including the previously described mutants of cyclin D1 that are defective in binding Cdk (K114E) and defective in binding the pRb protein GH (G7A/H8A) or T286A, a cyclin D1 mutant that cannot be phosphorylated by GSK-3β (63, 64) (Fig. 6A). Western blot analysis in our previous studies has confirmed similar levels of expression of the cyclin D1 mutants compared with wild type in transfected cells using the FLAG antibody (10). Mutation of the Cdk-binding domain in cyclin D1 (K114E) augmented p300 activity, suggesting the Cdk function of cyclin D1 was not necessary for repression of p300. In addition, the cyclin D1 GH mutant (G7A/H8A) and T286A mutants enhanced p300 activity (Fig. 6A). The cyclin D1 T286A mutant binds to Cdk4 but fails to form active Cdk. These studies suggest that the kinase activity of cyclin D1 is not required for repression of p300 and raise the possibility that the kinase activity may actually augment p300 activity.

In order to delineate the region of cyclin D1 that is involved in repression of p300 transactivation, a series of N-terminal deletion constructs of cyclin D1 was prepared (Fig. 6B, left panel). The efficiency of p300 repression by the cyclin D1 deletion mutants was compared with those of cyclin D1 wild type. Deletion of amino acids 143–178 of cyclin D1 abolished repression of p300 (Fig. 6B, right panel), indicating that amino acids 143–178 of cyclin D1 are involved in p300 repression. The
143–178 region of cyclin D1 is predicted to form a helix-loop-helix structure (10). Homology model studies suggested that the cluster of hydrophobic residues (amino acids 137–148, LLXXXLLLVXXL) in this region binds PPARγ/H9253 at the same region where co-repressors and co-activators bind to PPARγ/H9253. The current study suggests that the hydrophobic residues in 137–148 region of cyclin D1 are functionally important in interaction with and repression of the PPARγ co-activator p300 (10).

Molecular Genetic Phenotype of the Genes Co-regulated by Cyclin D1 and p300—The current studies demonstrate that cyclin D1 inhibits p300 activity which in turn may regulate a subset of gene functions. To determine the genes repressed by cyclin D1 and regulated by p300 at physiological levels, we introduced cyclin D1 into cyclin D1-deficient cells and determined the molecular genetic phenotype of cyclin D1-regulated genes by using microarray analysis. Comparison was made between cyclin D1-deficient cells infected with the retroviral vector virus and those infected with the cyclin D1 expression vector (Fig. 7A). Western blot analysis was conducted of the MEFs to ensure the levels of cyclin D1 expressed from the retroviral vector reflected physiological levels. Comparison was made between cyclin D1−/−/H11001 cyclin D1−/−/H11002 and cyclin D1+/#/H11002 MEFs. The levels of cyclin D1 were similar in the cyclin D1 expression vector and wild type MEFs (Fig. 7B). Microarray analysis identified a subset of genes that was either repressed or induced by cyclin D1 (Fig. 7C, lanes 1–3 versus 4–6). As we have shown that cyclin D1 inhibited p300 transactivation function, we next identified those genes regulated by p300 (Fig. 7C, lanes 7–9 versus 10–12). A subset of genes that was induced by cyclin D1 and reciprocally regulated by p300 includes genes involved in maintaining DNA replication fidelity, such as Mcm3 (minichromosome maintenance-deficient 3), Mcm4, and Rfc4 (replication factor C) (Table I).

Consistent with the role for cyclin D1 as an inhibitor of differentiation and a promoter of cellular proliferation and migration, expression of cyclin D1 inhibited cell cycle inhibitory proteins, including RASSF1 (Ral GDS/AF6) and cyclin-dependent kinase inhibitor 2B (p15), LCN2 (lipocalin), thrombospondin 1, Foxg1 (forkhead box G1), and Dkk2 (Dickkopf homolog 2). Conversely, cyclin D1 induced several pro-proliferative proteins (cyclin F), polo-like kinase 4, ECT2 oncogene (Ect2), thymidine kinase 1, Cdc7 (cell division cycle-associated 7), thymidylate synthase, Cdc6 (cell division cycle 6 homolog), and Egr2 (early growth response 2). A subset of genes that was induced by cyclin D1 and reciprocally regulated by p300 includes genes involved in maintaining DNA replication fidelity, including Mcm3, Mcm4, and Rfc4 (replication factor C) (Table I).

To determine whether the altered gene expression profile observed by microarray was recapitulated at the level of direct promoter interactions, a subset of genes was further assessed for repression by cyclin D1 and induction by p300. PPARγ-responsive genes were repressed by cyclin D1, consistent with previous studies (45). Several genes repressed by cyclin D1 and induced by p300 promoted cellular differentiation and cell cycle.
Repression of p300 Transactivation by Cyclin D1

Fig. 4. Repression of p300 by cyclin D1 requires the CH1, bromo, and CH2 domains of p300. Gal4-p300 (A and B) or E2-p300 (C, D, and E) expression vectors shown schematically were assessed for repression by cyclin D1. Data are shown as relative light units (D) or as percent repression (B and E) (mean ± S.E.) for >9 separate experiments. WT, wild type.
Dkk1 (Dickkopf 1) is an inhibitor of Wnt signaling that depends upon LRP-6 and functions to inhibit endogenous Wnt (65). Consistent with mRNA expression data, cyclin D1 reduced Dkk1 promoter activity by 50% (Fig. 8A, lane 1 versus 2), whereas p300 induced Dkk1 promoter activity by 10-fold (Fig. 8A, lane 3 versus 4). Cyclin D1 inhibited p300-dependent induction of Dkk1 by 25% (Fig. 8A, lane 5 versus 6).

The anti-angiogenic factor thrombospondin-1 (Tsp-1) was the first naturally occurring inhibitor of angiogenesis to be identified (66). Tsp-1, which inhibits MMP-9 activity and inhibits angiogenesis by binding to the CD36 receptor present on endothelial cells (67, 68), is repressed by several oncogenes, including Ras and c-Myc (69). Consistent with these signaling pathways, cyclin D1 repressed Tsp-1 promoter activity by 45% (Fig. 8B, lane 1 versus 2). p300 induced Tsp-1 activity 4-fold (Fig. 8B, lane 3 versus 4). p300-dependent induction of Tsp-1 was repressed by cyclin D1 by 60% (Fig. 8B, lane 5 versus 6).

Expression of FoxG1, a member of the Forkhead family that induces cellular differentiation and cell cycle arrest (70), was repressed by cyclin D1 and induced by p300. Forkhead proteins are known to inhibit cyclin D1 expression. To determine whether cyclin D1 inhibits Forkhead function further, the canonical Forkhead-binding site was used in heterologous reporter assays. Consistent with the mRNA findings, cyclin D1 inhibited FKHR signaling (Fig. 8C). Consistent with findings that CBP/p300 induces the FOXO1 and DAF6 involving the CH domain (71), p300 enhanced FKHRLUC activity up to 8-fold.
transfection (Fig. 9, right panel) (n = 6).

Several genes induced by cyclin D1 are also induced by interferon (Table I). To determine whether cyclin D1 induced interferon-dependent signaling, a multiple synthetic response element was used (GAS, LUC) (40). Cyclin D1 induced multimeric GAS, LUC reporter activity, as did p300 (Fig. 8D), consistent with previous studies that p300 activates interferon signaling. Several viral control promoters (CMV and SV40) were not affected by cyclin D1 (59). Together, these studies identify the molecular genetic phenotype of cyclin D1-repressed genes that are also regulated by p300.

Cyclin D1 Inhibits p300 HAT Activity in Vitro—These studies demonstrated cyclin D1 co-associated with p300 in immune complexes and repressed p300 activity through the bromo and CH domains. The bromo domain binds to acetylated lysine residues and augments acetylation of target substrates (18, 72, 73). It would be predicted that cyclin D1 may inhibit p300-mediated substrate binding and/or acetylation. To examine the possibility that cyclin D1 inhibition of p300 activity through the bromo domain may in turn inhibit the ability of p300 to acetylate target substrates, including autoacetylation of p300 and acetylation of core histones, in vitro HAT assays were conducted. HEK 293 cells were co-transfected with expression vector for p300 and either an expression vector for cyclin D1 or vector control. Immunoprecipitation of p300 from the transfected cellular extracts was conducted as a source of HAT, and increasing amounts of immunoprecipitate were used in HAT assays with the histone mixture H2A/B, H3, and H4 as substrates for p300 enzyme activity. Analysis of p300 autoacetylation and p300-dependent acetylation of histones was compared by using the cellular extracts (Fig. 9). Collectively, these studies demonstrated that cyclin D1 expression inhibits autoacetylation of p300 (Fig. 9, A and B) and inhibits acetylation of core histones by p300 in cultured cells (Fig. 9, C and D). These studies raise the possibility that the inhibition of p300 acetylation by cyclin D1 may contribute to cyclin D1-dependent transcriptional repression of p300.

**DISCUSSION**

p300 is a modular protein in which multiple distinct surfaces interact with regulatory proteins. Here cyclin D1 associated with p300 in immunoprecipitation assays, co-associated at target promoters in the context of local chromatin in ChIP assays, and repressed p300 trans-activity. p300 activity was regulated up to 60-fold by cyclin D1. Repression of p300 involved the CH1, CH3, and bromo domain. The region of repression within p300 was distinct from the domain regulated by p21cip1/waf1 (23, 24, 54, 74). The p21cip1/waf1 induction of p300 involves de-repression through the CRD1 domain. p21cip1/waf1 and cyclin D1 have therefore opposing effects on p300 activity through distinct domains. p21cip1/waf1 and cyclin D1 have been shown previously to regulate opposing effects of cell cycle progression and tumorigenesis in response to the Ras oncogene (75, 76). Thus, the current observations extend the previously defined interactions between cyclin D1 and p21cip1/waf1 by identifying their interaction with the histone acetyltransferase p300.

The abundance and activity of p300 are limited in the activity of many transcription factors. Components impacting p300 activity are assessed by the Gal4 system, including β-catenin, atrophin1, ATF-2, SIRT1, and p53, which regulate p300 activity up to 3-fold (20, 43, 46–50, 53). p300 function is regulated by several different enzymes and kinases, including acetylases and deacetylases (25). For example, SIRT1 inhibits p300 function, providing evidence for cross-talk between the NAD-dependent deacetylases (SIRT1) and histone acetyltransferase p300 (53). The abundance of cyclin D1 is tightly regulated by oncoproteins and growth factor signals. Thus the inhibition of p300 by cyclin D1 may provide a mechanism to integrate mitogenic or oncogenic signals with a subset of target genes that are regulated by p300.

The bromo domain of p300 was required for cyclin D1-dependent repression. Bromo domains bind specifically to acetylated lysine residues of histones and non-histone proteins such as p53 (18, 77). It has been proposed that bromo domains in HAT proteins enhance the off rate of the catalytic domain, while hindering HAT activity to chromatin, thereby enhancing
FIG. 7. Molecular genetic phenotype of p300 and cyclin D1-dependent signaling. A, schematic representation of MEFs analyzed. B, Western blot analysis for cyclin D1 with vinculin as loading control; C, hierarchical clustering of microarray analysis from p300+/+ and p300−/− MEFs, cyclin D1−/− MEFs, infected either with MSCV-cyclin D1-IRESGFP or MSCV-IRESGFP control virus.
## Table 1

**Genes co-regulated by cyclin D1 and p300**

| Cyclin D1 vs. vector | Fold change | p300+/+ vs. p300−/− | Fold change | Gene symbol | annotations | Gene title annotations |
|----------------------|-------------|---------------------|-------------|-------------|-------------|------------------------|
| 1 Down 3.91 Up 10.80 | Kitsl | Kit ligand |  | | | |
| 2 Down 6.72 Up 80.49 | Thy1 | Thymus cell antigen 1, θ | | | | |
| 3 Down 2.11 Up 61.29 | Mrpplf4 | Mitogen-regulated protein, proliferin 4 | | | | |
| 4 Down 2.01 Up 21.53 | Pif | Proliferin | | | | |
| 5 Down 3.71 Up 11.52 | Serpinf1 | Serine (or cysteine) proteinase inhibitor, clade F |  | | | |
| 6 Down 3.73 Up 18.14 | Dkk2 | Dickkopf homolog 2 (Xenopus laevis) | | | | |
| 7 Down 2.27 Up 6.22 | Foxg1 | Forkhead box G1 | | | | |
| 8 Down 2.56 Up 8.72 | Tnfrsf11b | Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) | | | | |
| 9 Down 3.25 Up 2.73 | Adm | Adrenomedullin | | | | |
| 10 Down 3.78 Up 2.17 | Fze1 | Fascladation and elongation protein ζ1 | | | | |
| 11 Down 2.15 Up 2.04 | C630002M10Rik | RIKEN cDNA C630002M10 gene | | | | |
| 12 Down 10.40 Down 381.95 | Cnn1 | Calponin 1 | | | | |
| 13 Down 2.21 Down 4.02 | Mad4 | Max dimerization protein 4 | | | | |
| 14 Down 8.79 Down 17.21 | Tll | Toll-like | | | | |
| 15 Down 2.60 Down 5.15 | F3 | Coagulation factor III | | | | |
| 16 Down 5.18 Down 2.55 | Ccd2 | Cdkn2 | | | | |
| 17 Down 6.44 Down 2.03 | Fhl1 | Four and a half LIM domains 1 | | | | |
| 20 Down 2.72 Down 3.20 | D7Ertd458e | DNA segment, Chr 7, ERATO Doi 458, serine (or cysteine) proteinase inhibitor, clade E | | | | |
| 21 Down 4.80 Down 12.62 | Serpin 1 | | | | | |
| 22 Down 3.91 Down 5.95 | Hist1h2bc | Histone 1, H2bc | | | | |
| 23 Down 3.99 Down 4.74 | Tagln | Transgelin | | | | |
| 24 Down 5.54 Down 3.02 | Ctgf | Connective tissue growth factor | | | | |
| 25 Down 3.17 Down 6.08 | Pprz1 | Protein-tyrosine phosphatase, receptor type Z | | | | |
| 26 Down 2.35 Down 4.29 | Strm | Striain | | | | |
| 27 Down 3.31 Down 39.95 | Crfl1 | Cytokine receptor-like factor 1 | | | | |
| 28 Down 2.12 Down 20.74 | Lcn2 | Lipocalin 2 | | | | |
| 29 Down 2.10 Down 3.72 | Thbs1(Tap1) | Thrombospondin 1 | | | | |
| 30 Down 2.91 Down 2.63 | 1110029F20Rik | RIKEN cDNA 1110029F20 gene | | | | |
| 31 Down 2.09 Down 4.02 | Sorbs1 | Sorbin and SH3 domain containing 1 | | | | |
| 32 Down 2.34 Down 2.70 | AfiS2838 | UAI-M-BH0-ak-b-10-0-UAI.s1 | | | | |
| 33 Down 2.36 Down 3.81 | Nuprl | Nuclear protein 1 | | | | |
| 34 Down 2.25 Down 3.32 | Hmx1 | Heme oxygenase (decelycling) 1 | | | | |
| 35 Down 2.22 Down 7.28 | D5Ertd593e | DNA segment, Chr 5, ERATO Doi 593 | | | | |
| 36 Down 4.34 Down 2.50 | Bdnf | Brain-derived neurotrophic factor | | | | |
| 37 Down 17.73 Down 29.50 | Saa3 | Serum amyloid A3 | | | | |
| 38 Down 3.00 Down 2.45 | Rassfl | Ras association (RulGDS/AF-6) domain family 1 | | | | |
| 39 Down 2.38 Down 2.23 | Ankrd1 | Ankyrin repeat domain 1 (cardiac muscle) | | | | |
| 40 Down 2.47 Down 2.82 | Cdkn2b | Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) | | | | |
| 41 Up 3.42 Up 3.68 | Uhrf1 | Ubiquitin-like, containing PHD and RING finger | | | | |
| 42 Up 2.31 Up 7.05 | Ccnf | Domains, 1 cyclin F | | | | |
| 43 Up 2.20 Up 3.03 | Flk4 | Polo-like kinase 4 (Drosophila) | | | | |
| 44 Up 2.09 Up 2.29 | Snpd3 | Small nuclear ribonucleoprotein D3 | | | | |
| 45 Up 2.49 Up 2.17 | Anpase | Acid (leucine-rich) nuclear phosphoprotein 32 family, member E | | | | |
| 46 Up 2.08 Up 10.42 | Ect2 | Oncogene | | | | |
| 47 Up 2.85 Up 2.46 | S4303423A01Rik | RIKEN cDNA S4303423A01 gene | | | | |
| 48 Up 2.73 Up 2.86 | Mgst3 | Microsomal glutathione S-transferase 3 | | | | |
| 49 Up 2.13 Up 5.78 | Tk1 | Thymidine kinase 1 | | | | |
| 50 Up 2.41 Up 4.95 | A730011O11Rik | RIKEN cDNA A730011O11 gene | | | | |
| 51 Up 2.22 Up 2.29 | Cdc57 | Cell division cycle-associated 7 | | | | |
| 52 Up 2.37 Up 10.44 | Cdkn3 | Cyclin-dependent kinase inhibitor 3 | | | | |
| 53 Up 4.69 Up 8.57 | Gsa1 | Growth arrest specific 1 | | | | |
| 54 Up 2.30 Up 4.21 | D030034H08 | Hypothetical protein D030034H08 | | | | |
| 55 Up 2.18 Up 4.50 | 2810417H13Rik | RIKEN cDNA 2810417H13 gene | | | | |
| 56 Up 2.03 Up 2.35 | Zfp362 | Zinc finger protein 36, C3H type-like 2 | | | | |
| 57 Up 6.80 Up 14.94 | Itln1 | Interleukin 1 receptor antagonist | | | | |
| 58 Up 2.60 Up 2.86 | Cof6a2 | Procollagen, type VI, α2 | | | | |
| 59 Up 3.62 Up 7.33 | Lmo7 | LIM domain only 7 | | | | |
local chromatin acetylation (18). Nuclear magnetic resonance spectroscopy demonstrated the physical association of the bromo domain from p300/CBP-associated factor and histone-derived peptides containing acetylated lysine (72). Bromo domains exhibit a left-hand twisted four-helical bundle, with two loops at one end of the bundle forming a hydrophobic lysine-binding pocket that selects acetyl-lysine rather than the charged unmodified lysine (78). Additional specificity of the bromo domain is derived from residues flanking the acetylated lysine in a given target protein (77). The bromo domain serves as a selective signaling module that is induced in response to physiological and pathological activities. Physical interaction between cyclin D1 and p300 may modulate p300 bromo domain interactions with its target proteins, thereby regulating these physiological responses. Thus, the CBP bromo domain was recently shown to bind a lysine-acetylated p53 peptide through specific interaction that is required for the activity of p53 in response to ultraviolet-induced DNA damage (77). Cyclin D1 abundance is tightly regulated in expression (10, 11, 45). Interactions between cyclin D1 and p300 may regulate acetylated lysine residues on histones and non-histone target proteins to regulate expression of p300 target genes and to modulate local chromatin remodeling complexes.

p300 regulates the activity of many transcription factors. To analyze the functional significance of cyclin D1-dependent regulation of p300, we conducted a genome-wide analysis of cyclin D1 and p300-regulated genes. We thereby determined the p300-regulated genetic targets shared with cyclin D1. Molecular genetic profiling identified a signature of genes that was both repressed by cyclin D1 and induced by p300 (Fig. 7 and Table I). Conversely, a subset of genes induced by cyclin D1 was also regulated by p300. This subset of genes promoted cell proliferation, DNA synthesis, and DNA replication.

It is of interest that a subset of genes repressed by cyclin D1 and induced by p300 plays a role in cellular differentiation. Dkk2 expression was reduced by cyclin D1 and induced by p300 at the mRNA and promoter level. Dkk1 is known to inhibit Wnt signaling, correlating with its ability to bind the LRPS receptor (79). Dkk inhibits β-catenin Tcf signaling (65). Thus, Dkk functions as an extracellular inhibitor to fine-tune spatial and temporal patterns of Wnt activity through regulating autocrine Wnt signaling in human cancer cells (80). Cyclin D1 is induced by activation of the Wnt/β-catenin signaling pathway (81) through induction of Wnt (81–83) and activating β-catenin mutants (81) or downstream component, including integrin-linked kinase (81, 83). Furthermore, cyclin D1 is required for adenomatous polyposis of the colon (Ap-c)-induced colonic tumorigenesis (45). Thus, cyclin D1 serves as both a downstream target of Wnt signaling and as an upstream regulator of Wnt signaling through regulation of Dkk. The finding that cyclin D1 regulates Dkk expression and promoter activity is consistent with growing evidence that cyclin D1 functions through autocrine mechanisms to regulate cellular function (8).

For example, cyclin D1 is known to regulate expression of several secreted proteins such as ACRP30 (10), which are involved in angiogenesis (84). Cyclin D1 inhibited p300-dependent induction of KHR signaling. Forkhead transcription factors are involved in differentiation of erythropoietin stem cell factor-mediated signaling in primary erythroid progenitors (85). Along with FoxG1, the expression of a number of genes that induce cellular growth arrest was inhibited by cyclin D1 (RassF1, Cdkn2b, Mad4, and Tsp1), consistent with the known pro-proliferative function of cyclin D1.

Cyclin D1 repression of p300 and PPARγ activity was TSA-dependent and augmented by histone deacetylases. p300 activity was 7-fold greater in cyclin D1−/− cells, and the difference

---

**Table I—continued**

| Cyclin D1 vector | Fold change | p300-/+ vs. p300-/- | Fold change | Gene symbol | Gene title | annotations |
|------------------|-------------|---------------------|-------------|-------------|-----------|-------------|
| 60               | Up          | 3.32                | Up          | 6.74        | Hmgb2     | High mobility group box 2 |
| 61               | Up          | 2.09                | Up          | 3.91        | Tyms      | Thymidylate synthase |
| 62               | Up          | 2.13                | Up          | 2.07        | Mcm4      | Minichromosome maintenance-deficient 4 |
| 63               | Up          | 2.68                | Up          | 2.52        | H2afx     | Homolog (Saccharomyces cerevisiae) |
| 64               | Up          | 2.45                | Up          | 2.42        | Lig1      | Ligase I, DNA, ATP-dependent |
| 65               | Up          | 2.12                | Up          | 2.46        | Fgfn1     | Fidgetin-like 1 |
| 66               | Up          | 2.81                | Up          | 2.59        | Refp1     | RNA and export factor binding protein 1 |
| 67               | Up          | 2.81                | Up          | 2.43        | Lmb1      | Lamin B1 |
| 68               | Up          | 2.06                | Up          | 2.19        | Mcm3      | Minichromosome maintenance-deficient 3 (S. cerevisiae) |
| 69               | Up          | 2.48                | Up          | 7.20        | D17H8S56E-5 | DNA segment, Chr 17, human D6S56E 5 |
| 70               | Up          | 2.32                | Up          | 2.09        | Cdc6      | Cell division cycle 6 homolog (S. cerevisiae) |
| 71               | Up          | 2.61                | Up          | 3.19        | Rfc6      | Replication factor C (activator 1) 4 |
| 72               | Up          | 2.31                | Up          | 5.30        | 4432406C08Rik | RIKEN cDNA 4432406C08 gene |
| 73               | Up          | 3.26                | Up          | 6.24        | 4432406C08Rik | RIKEN cDNA 4432406C08 gene |
| 74               | Up          | 2.27                | Up          | 6.20        | Ttk       | Trk protein kinase |
| 75               | Up          | 2.58                | Up          | 10.35       | Pitx2     | Paired-like homeodomain transcription factor 2 |
| 76               | Up          | 2.06                | Up          | 2.89        | Smc41     | SMCM4 structural maintenance of chromosomes 4-like 1 (yeast) |
| 77               | Up          | 3.39                | Up          | 9.38        | Birc5     | Baculoviral IAP repeat-containing 5 |
| 78               | Up          | 2.07                | Up          | 2.10        | Rbm14     | RNA-binding motif protein 14 |
| 79               | Up          | 2.28                | Up          | 2.12        | Mcm3      | Minichromosome maintenance-deficient 3 (S. cerevisiae) |
| 80               | Up          | 2.73                | Down        | 7.45        | Ogn       | Osteoglycin |
| 81               | Up          | 2.45                | Down        | 4.41        | Gip2      | Interferon, α-inducible protein |
| 82               | Up          | 3.66                | Down        | 2.66        | Csp2      | Cysteine- and glycine-rich protein 2 |
| 83               | Up          | 2.44                | Down        | 10.24       | Gip2      | Interferon, α-inducible protein |
| 84               | Up          | 2.88                | Down        | 11.59       | Ogn       | Osteoglycin |
| 85               | Up          | 4.46                | Down        | 6.32        | Egr2      | Early growth response 2 |
| 86               | Up          | 2.32                | Down        | 6.32        | Csf       | Complement component factor h |
| 87               | Up          | 2.48                | Down        | 2.40        | AA816121 | Diabetic nephropathy-related gene 1 mRNA, partial sequence |
in activity between cyclin D1\(^{+/+}\) and cyclin D1\(^{-/-}\) cells was TSA-reversible. Cyclin D1 repression of p300 involved TSA-sensitive histone deacetylases consistent with recent findings that cyclin D1 physically associates with and recruits HDACs to promoters in the context of their local chromatin structure (11).

Cyclin D1, in addition to binding cyclin-dependent kinases 4 and 6 (Cdk4/Cdk6) and pRb, forms physical associations with p300, P/CAF (p300/CBP associated factor), Myb, MyoD, and the cyclin D1 myb-like binding protein (DMP1) (reviewed in Refs. 8, 59, 86–88). Cyclin D1 and D2 were capable of inhibiting MyoD (71). v-Myb was preferentially repressed by cyclin D1 (87). Although cyclin D2 and D3 were also capable of repressing v-Myb, the helix-loop-helix-like protein DMP1 was repressed by each of the D-type cyclins (86). In contrast, the repression of BETA2/NeuroD by cyclin D1 was not reconstituted by either cyclin D2 or D3 (39). Previous studies have demonstrated a role for cyclin D1 in repression of several distinct transcription factors (8). Typically, the Cdk4-binding domain of cyclin D1 was not required for transcriptional repression. Here, cyclin D1 repression of p300 transactivation was Cdk-independent and may contribute to several of the previously identified transcriptional regulatory functions of cyclin D1.
FIG. 9. Cyclin D1 inhibits HAT activity of p300 in vitro. HEK 293 cells were co-transfected with an expression vector for p300 and either with expression vector for cyclin D1 or vector control. The cellular lysates were immunoprecipitated with a p300 antibody. Increasing amounts of the immune complexes (10, 20, 40, and 80 µl) were incubated with a histone mixture (H2A/B, H3, and H4) in the presence of [14C]acetyl-CoA at 30 °C for 1 h. The reactions were dissolved in 12% SDS-PAGE followed by radiography. A, autoacetylation of p300 in the absence (lanes 1–4) or presence (lanes 5–8) of cyclin D1. B, quantitative presentation of p300 autoacetylation. Densitometry of [14C]acetly incorporation is shown in arbitrary units. C, acetylation of core histones by p300 is inhibited by cyclin D1. Incorporation of [14C]acetyl group into histones by p300 was compared in the absence or presence of cyclin D1. Bottom panel, shorter exposure of the radiography is shown. D, quantitative presentation of histone acetylation by p300. Densitometry of [14C]acetyl incorporation into histone substrates is shown in arbitrary units.
