Activation of the cyclin D1 Gene by the E1A-associated Protein p300 through AP-1 Inhibits Cellular Apoptosis*

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The adenovirus E1A protein interferes with regulators of apoptosis and growth by physically interacting with cell cycle regulatory proteins including the retinoblastoma tumor suppressor protein and the coactivator proteins p300/CBP (where CBP is the CREB-binding protein). The p300/CBP proteins occupy a pivotal role in regulating mitogenetic signaling and apoptosis. The mechanisms by which cell cycle control genes are directly regulated by p300 remain to be determined. The cyclin D1 gene, which is overexpressed in many different tumor types, encodes a regulatory subunit of a holoenzyme that phosphorylates and inactivates PRB. In the present study E1A12S inhibited the cyclin D1 promoter via the amino-terminal p300/CBP binding domain in human choriocarcinoma JEG-3 cells. p300 induced cyclin D1 protein abundance, and p300, but not CBP, induced the cyclin D1 promoter. cyclin D1 or p300 overexpression inhibited apoptosis in JEG-3 cells. The CH3 region of p300, which was required for induction of cyclin D1, was also required for the inhibition of apoptosis. p300 activated the cyclin D1 promoter through an activator protein-1 (AP-1) site at −954 and was identified within a DNA-bound complex with c-Jun at the AP-1 site. Apoptosis rates of embryonic fibroblasts derived from mice homozygously deleted of the cyclin D1 gene (cyclin D1−/−) were increased compared with wild type control on several distinct matrices. p300 inhibited apoptosis in cyclin D1−/− fibroblasts but increased apoptosis in cyclin D1+/− cells. The anti-apoptotic function of cyclin D1, demonstrated by sub-G1 analysis and annexin V staining, may contribute to its cellular transforming and cooperative oncogenic properties.

During the mid to late G1 phase of the cell cycle, the mammalian cell encounters a critical point where an irrevocable commitment takes place to either enter replicative DNA synthesis or to withdraw from the cell cycle with an unduplicated genome. This critical G1 checkpoint, known as the restriction or R point (1), separates the cell cycle into an early mitogen-dependent period and a subsequent primarily autonomous phase leading to cellular division (2). Determining the mechanisms regulating passage through the R point is important to the understanding of diverse cellular processes including cellular proliferation, cell cycle arrest allowing for DNA repair, terminal differentiation, and apoptosis (2–4). The precise regulation of early G1 phase events is mediated by the G1 phase cyclins and their inhibitors together with their substrates, including the retinoblastoma (pRB) tumor suppressor protein. Phosphorylation and inactivation of pRB is conveyed by serine/threonine kinase holoenzymes, composed of a regulatory G1 cyclin of the D family and its associated catalytic subunit Cdk4 or Cdk6 (2). Immunoneutralizing and antisense experiments have demonstrated cyclin D1 abundance conveys a rate-limiting role in promoting progression through the early G1 phase induced by serum addition, growth factors, and mitogens such as estrogen (5–8).

Consistent with a role in promoting early G1 phase progression, cyclin D1 expression and gene transcription is induced in early G1 by proliferative growth factors through members of the mitogen-activated protein kinase pathway (9–13). Cyclin D1 is transcriptionally induced by diverse oncogenic signals including members of the Ras (p21cc), Rac1, RhoA), and Dbl (Dbl, Ect) superfamilies, pp60c-src, and the β-catenin/APC pathway (10, 14–19). Cyclin D1 is overexpressed in an array of tumor types, and mammary tumors were induced by transgenic overexpression of cyclin D1 in the murine mammary glands, consistent with an oncogenic role for cyclin D1 (20). Mice homozygously deleted of the cyclin D1 gene are smaller than normal (21, 22), with a proliferative defect of their embryonic fibroblasts (23) and increased retinal apoptosis (24).

In addition, cyclin D1 conveys a G1/S checkpoint function by blocking unregulated S phase entry in the presence of DNA-damaging agents (6, 25). The growth inhibitory effects of cyclin D1 have been observed in MCF-10F and several other mammary epithelial cells (HBL-100, HC11, 184B5) (26). Thus cyclin D1 accelerates transit through G1 but inhibits S phase traversal when moderately up-regulated (5, 6). In murine cells,
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Inhibition of cellular apoptosis. Cyclin D1 expression is induced during p53-mediated cell cycle arrest (27–29). The p53-mediated cell cycle arrest was overcome by Cdk sense in one study (30) and by antisense cyclin D1 mRNA in another (28) suggesting complex interactions between p53 and cyclin D1 function. Several oncogenes can induce p53, increasing apoptosis and/or premature senescence (31–33). The DNA adenovirus E1A induces p53 and promotes apoptosis (31, 34). The two domains of E1A that function to promote apoptosis include the pRB inactivating and the p300/CBP binding domains (35), and E1A induction of apoptosis is inhibited by p300 (36). The abundance of cyclin D1 protein is negatively regulated by E1A12S (29, 37, 38). It was proposed that the inhibition of cyclin D1 protein levels by E1A represented a function evolved by E1A to overcome the G1/S checkpoint function of cyclin D1 that blocks unregulated S-phase entry and apoptosis (29, 37, 38). It was not known whether the cyclin D1 gene was a direct transcriptional target of E1A nor were the mechanisms by which E1A regulated cyclin D1 transcription known.

The mechanisms by which p300/CBP and histone acetyltransferase activity modulate the cell cycle regulatory apparatus are complex. Microinjection of p300/CBP antibodies blocked transcription of immediate early genes that have been strongly implicated in mitogenic signaling (39). Recent studies in which the murine p300 and cbp genes were deleted demonstrated an important role for p300 in development and the enhancement of cellular proliferation (40). The embryos of p300-deficient mice displayed reduced bromodeoxyuridine uptake, and the embryonic fibroblasts derived from the mice (MEFs) showed a markedly reduced proliferation rate (40). Ribozyme ablation of p300 or CBP demonstrated distinct roles for p300 and CBP in cell cycle control and differentiation and suggested a requirement for p300 in cellular proliferation (41). These studies indicated that the identification of direct transcriptional targets of p300/CBP involved in cell cycle progression would be critical for understanding p300/CBP function in different cell types.

In the current studies, the amino-terminal p300/CBP binding domain of E1A was required for repression of cyclin D1 and cyclin E, but E1A-induced cyclin A, p300, but not CBP, directly augmented cyclin D1 promoter activity and protein abundance (42). The region of p300 that bound c-Jun/c-Fos was required for induction of cyclin D1. The overexpression of either cyclin D1 or p300 inhibited apoptosis; however, the ability of p300 to affect apoptosis was abolished in cells derived from mice homozygously deleted of the cyclin D1 gene. These findings indicate that the induction of the cyclin D1 gene by p300 may play an important role in the inhibition of cellular apoptosis.

MATERIALS AND METHODS

Construction of Reporter Genes and Expression Vectors—A series of 5′ promoter deletions and heterologous reporter constructions derived from the human cyclin D1 genomic clone linked to the luciferase reporter gene (10, 11), the cyclin A promoter luciferase reporter construct (43), the c-fos LUC reporter (12), the c-fos LUC reporter (43), and the reporter p3TPLUX (a gift from Dr. J. Massague) (44) which consists of trimeric AP-1 site linked to the E4 minimal promoter were previously described (45, 46). The pCMVHA p300 plasmids (47) (gifts from Dr. D. Livingston, Dr. R. Echner and Dr. S. Bhattacharya) were previously described. The expression plasmid, p300Δ1514-1922, functions as a p300-negative dominant mutant (48), and p300Δ1737-1836 deletes the E1A, PCAF binding region of p300, p300Δ1419-1721 deletes the region associated with histone acetyltransferase activity in vitro (49), and p300A1α502-1138 deleted amino acids corre-
natural occurring splice variant of E1A13S that removes conserved region 3 (CR3), mutants of E1A12S, and the protein binding domains disrupted by the mutations is shown (Fig. 2A). Previous studies performed in JEG-3 cells had demonstrated that the expression levels of the mutant E1A expression plasmids are very similar to wild type (46). The repression of −1745CD1LUC activity by E1A12S (2.7-fold, n = 27) is represented as 100%. The effects of specific mutation of E1A12S is shown as percent of 12 S values from at least 27 separate transfections. Mutation of the pRB binding domain in the E1A12S mutant, E1A12S m928, had relatively little effect on E1A12S-mediated repression of cyclin D1 (Fig. 2A), indicating that the mechanisms by which E1A12S inhibited cyclin D1 was not dependent upon interaction with p105 (Fig. 2A). The amino-terminal mutant (E1A12S RG2), which is incapable of binding p300, reduced repression of the promoter by greater than 90% (Fig. 2A). The E1A12S amino-terminal deletion mutant E1A12S A15–35 was also defective in repression, consistent with a role for p300 or related proteins in regulation of cyclin D1 (Fig. 2A). E1A12S YH47928, which abolishes binding to RB family members but retains p300 binding, was partially defective in transcriptional repression, consistent with previous studies that show that the RB-related family member proteins, p107 and p130, can bind the cyclin D1 promoter (57). The E1A mutants E1A12S A38–67 and E1A12S RG2/928, which are defective in both p300 and RB protein family binding, resulted in slight induction of reporter activity and are shown as 0% repression (Fig. 2A). The repression of cyclin D1 by E1A12S was dose-dependent (data not shown).

Analysis was next performed with the E1A12S mutants in the context of the cyclin E and cyclin A promoters. The repression of the cyclin E promoter by E1A12S was abolished by the E1A12S RG2 mutant but was unaffected by the E1A12S m928 mutant (Fig. 2B). Although the cyclin A promoter was induced by E1A12S (Fig. 1A) as recently shown (69), there was no significant loss of induction with the amino-terminal mutant E1A12S RG2 (data not shown). These findings are consistent...
ent with a role for the amino terminus of E1A12S in negative regulation of both the cyclin D1 and cyclin E promoters. Because the E1A12S RG2 mutant abolished 80% the E1A12S-mediated repression of the cyclin D1 promoter, the effect of overexpressing p300 on E1A12S-mediated repression was assessed. Increasing amounts of p300 overcame the inhibition of cyclin D1 by E1A12S in a dose-dependent manner (Fig. 2C). These studies suggested that the inhibition of cyclin D1 by E1A12S may be mediated in part by inhibition of p300 function.

**p300 Induces Cyclin D1 Protein Levels and Cyclin D1 Promoter Activity**—To examine the mechanisms by which p300 regulates cyclin D1 expression, transient expression studies were conducted in JEG-3 cells with the −1745CD1LUC reporter. Comparison was made with the effect of the empty expression vector cassette (Fig. 3A). p300 activated the cyclin D1 promoter 10-fold. (In these studies only 200 ng of expression vector was used in conjunction with 4.8 μg of reporter plasmid.) The induction of cyclin D1 by p300 was dose-dependent (not shown).

In recent studies a mutant of p300 was identified, p300Δ1514-1922, that functioned as a dominant negative mutant of p300-dependent activation of both p53- (48) and of AP-1-dependent transactivation. Overexpression of the p300Δ1514-1922 dominant negative expression plasmid reduced −1745CD1LUC reporter activity by 50–75% in a dose-dependent manner (Fig. 3B).

In order to determine whether p300 overexpression induced cyclin D1 protein levels, JEG-3 cells were transfected with the p300 expression vector, and Western blotting was performed after 24 h for p300 and for cyclin D1. cyclin D1 protein levels were induced 3-fold (Fig. 3C). The α-tubulin antibody demonstrated equal amounts of protein in each lane. The effect of p300 on cyclin D1 protein levels was next performed in cultured JEG-3 cells by co-transfection of p300 and a green fluorescent protein (GFP) expression vector, pEGFP-N1, to enrich for transfected cells. After cell sorting, to enrich for a pool of cells containing the expression vector (as described previously (51)), Western blotting with the p300 antibody, N15 (Santa Cruz Biotechnology), showed that p300 protein levels were increased 5-fold in p300-transfected JEG-3 cells (Fig. 3D, upper panel). Western blotting on these cell extracts with the cyclin D1 antibody, HD11, showed cyclin D1 protein levels were increased 4.5-fold in the p300-transfected cells (Fig. 3D, lower panel).

**Specific Co-integrator Proteins Differentially Regulate the Cyclin D1 Gene**—p300 and CBP have partial functional redun-

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*Fig. 2. The amino-terminal region of E1A12S is required for full inhibition of cyclin D1. A, a series of E1A12S mutants and their protein binding properties are shown schematically. The effect of the E1A mutants on repression of the cyclin D1 promoter is shown compared with the repression by E1A12S in JEG-3 cells with 12 S-mediated repression being set at 100%. The E1A12S mutants were co-transfected with the cyclin D1 promoter (4.8 μg) using increasing concentrations of expression plasmid (150, 300, and 600 ng). The E1A12S RG2 mutant fails to repress the cyclin D1 promoter. The data are means of at least 20 separate transfection for each plasmid shown. B, the effect of the E1A12S and the RG2 mutant on cyclin E promoter activity. The results are shown as the mean ± S.E. of five separate transfections. C, the effect of increasing amounts of p300 on the E1A12S-mediated repression of −1745CD1LUC reporter activity. Increasing amounts of p300 overcome the repression by E1A12S.* 

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*Fig. 3. p300 induction of cyclin D1 protein levels and promoter activity. A, p300 induces cyclin D1. p300 (300 ng) was transfected with 4.8 μg of −1745CD1LUC, and the fold induction shown is in comparison to equal amounts of empty expression vector cassette (CMV). B, the p300 dominant negative expression vector (150 or 300 ng) was transfected with the −1745CD1LUC reporter (2.4 μg). Data are shown as n = 32 separate transfections. C, JEG-3 cells were transfected with the p300 expression vector (lane 1) or an empty expression vector cassette (lane 2). D, the p300 expression vector was transfected together with the GFP plasmid pEGFP and GFP. FACS sorting of transfected cells was performed to enrich for p300-transfected cells (51). Western blotting was performed with the cyclin D1 antibody HD11 or the p300 antibody (N15, SC584, Santa Cruz Biotechnology).*

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*2 M. D’Amico, unpublished observations.*
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The CH3 region of p300 is required for induction of cyclin D1. A, schematic representation of the wild type and mutant p300 expression vectors in which the bromo domain (Δ1032–1138), the histone acetylase domain (Δ1419–1721), and the CH3 region (Δ1737–1836) were deleted. a.a., amino acid. B, transfections were conducted with the wild type or mutant p300 plasmids and the cyclin D1 promoter (-1745CD1LUC). Luciferase activity is shown compared with the wild type set at 100%. The p300 mutant (Δ1737–1836) is defective in activation of cyclin D1.

Fig. 5. The CH3 region of p300 is required for induction of cyclin D1. A, schematic representation of the wild type and mutant p300 expression vectors in which the bromo domain (Δ1032–1138), the histone acetylase domain (Δ1419–1721), and the CH3 region (Δ1737–1836) were deleted. a.a., amino acid. B, transfections were conducted with the wild type or mutant p300 plasmids and the cyclin D1 promoter (-1745CD1LUC). Luciferase activity is shown compared with the wild type set at 100%. The p300 mutant (Δ1737–1836) is defective in activation of cyclin D1.

Fig. 4. CBP does not induce cyclin D1 but induces retinoic acid signaling. A, a dose response was performed in JEG-3 cells with -1745CD1LUC, and increasing amounts of CBP or p300 was performed, and the fold change in activity of the cyclin D1 promoter was assessed. Comparison was made between equal amounts of the CBP or p300 expression plasmid and the empty expression vector cassette. B, the retinoic acid-responsive reporter DR5LUC was introduced into HeLa cells with the retinoid A receptor and retinoic acid-responsive reporter in a dose-dependent manner (Fig. 4). A dose response with increasing amounts of CBP was performed, and the fold change is shown for n = 6 separate experiments as mean ± S.E. The induction of the DR5LUC reporter by the addition of CBP is shown as recently described (70).

dancy in murine embryonic development (40). In a recent study the p21^{Cip1} promoter was selectively induced by p300, and the p27^{Kip1} promoter was induced by CBP (41). We therefore compared the activity of p300 and CBP using the cyclin D1 promoter. In contrast with p300, which induced cyclin D1 promoter activity 5-fold, CBP did not induce cyclin D1 at any of the doses assessed (Fig. 4A), even using up to 10-fold greater amounts of plasmid (not shown). By using Western blots of the epitope-tagged plasmids, the expression levels of p300 and CBP were found to be identical (not shown). In order to assess the activity of CBP, we next assessed the CBP expression vector in a previously described transactivation assay in HeLa cells (70). In keeping with these previous studies, overexpression of CBP enhanced retinoic acid-induced retinoid A receptor and retinoic acid-responsive reporter in a dose-dependent manner (Fig. 4B).

The CH3 Region of p300 Is Required for Full Induction of Cyclin D1—Studies of the p300 protein have identified several distinct functional domains (Fig. 5A). In order to identify domains of p300 that regulate cyclin D1 promoter activity, previously characterized expression plasmids encoding p300 mutants, deleted within either the bromo domain, histone acetyltransferase domain or the CH3 region, were assessed (Fig. 5A). The effects of the mutants were compared with the effect of equal amounts of wild type p300 and are shown as percent wild type induction (Fig. 5B). The p300 mutant Δ1032–1138 deletes the region homologous to the bromo domain. The Δ1032–1138 mutant retained 70% of wild type cyclin D1 induc-
CBP (lane 9). Hence, p300/CBP-immunoreactive material is contained in a specific DNA-bound protein complex with AP-1 at the cyclin D1 p300-responsive element. These findings are consistent with recent findings in which p300/CBP was found within a DNA-bound complex with p53 (48), although in the current experiments the p300 polyclonal antibody was used at 1/10th the concentration used in previous experiments to avoid possible nonspecific inhibition of DNA binding.

Because these studies indicated that c-Jun was a likely transcriptional target of p300 induction of cyclin D1, we examined the effect of the c-Jun dominant negative and c-Jun wild type on p300-induced cyclin D1 promoter activity (Fig. 6D). Comparison was made with equal molar amounts of co-transfected empty expression vector cassette for c-Jun. Low concentrations of c-Jun expression plasmid were used, as detailed under “Materials and Methods” (300–600 ng), to avoid spurious cross-squenching. The activity of the −1745 CD1LUC reporter in the presence of p300 was established as 100% in the figure. Overexpression of c-Jun further enhanced p300-induced activity of the cyclin D1 promoter by 207% (±12%, n = 12). Overexpression of the c-Jun dominant negative TAM-67 expression vector reduced p300-induced cyclin D1 promoter reporter activity in a dose-dependent manner to 64 ± 4% (n = 12) for 300 ng and 56 ± 3% (n = 12) for 600 ng, respectively. These studies are consistent with a role for c-Jun as an intermediary in p300 induction of the cyclin D1 promoter.

Cyclin D1 and p300 Inhibit Basal Cellular Apoptosis in Trophoblastic Cells—In previous studies the effect of cyclin D1 to either induce or inhibit cell cycle progression was cell type-dependent (5, 26). Furthermore, cyclin D1 has been shown to either promote or inhibit apoptosis. Analyses were therefore performed to examine the effect of cyclin D1 and p300 on apoptotic and cell cycle indices. Two independent methods were used to assess apoptosis. The percentage of cells with subdiploid DNA content (referred to as the sub-G₁ population), which represent apoptotic cells (53, 54) was assessed by FACS analysis, and annexin V staining was performed. Annexin V staining as a sensitive measure of early apoptosis (53, 54) was used to assess apoptosis. The percentage of cells with subdiploid DNA content (referred to as the sub-G₁ population), which represent apoptotic cells (53, 54) was assessed by FACS analysis, and annexin V staining was performed. Apoptotic cells were distinguished by PI staining as a sensitive measure of early apoptosis (53, 54) and allowed detection of GFP fluorescence in ethanol-treated cells and thereby cell cycle analysis (50). GFP-positive cells were analyzed for cell cycle distribution and for the percentage of cells with subdiploid DNA content (referred to as the sub-G₁ population), which represent apoptotic cells (53, 54). This method provides a highly sensitive measure of the effect of transfection expression plasmids on cell cycle and apoptotic parameters.

Cyclin D1 overexpression did not alter the distribution of cells in the G₁ or S phases (n = 3); however, there was a reduction in the proportion of apoptotic cells with subdiploid DNA content (Fig. 7A). The fraction of apoptotic cells was reduced approximately 30% by cyclin D1 overexpression (Fig. 7A). p300 overexpression reduced the fraction of apoptotic cells by 60%. Overexpression of the CH3 mutant, p300Δ1737–1836, did not inhibit apoptosis. Similar results were obtained in three separate experiments.

In order to examine further the role of basal cyclin D1 levels in cellular apoptosis, embryonic fibroblasts derived from either wild type mice or mice homozygously deleted of the cyclin D1 gene were analyzed. Our previous studies established that the cyclin D1−/− fibroblasts proliferated more slowly than wild type control cells (23). In the current studies the apoptotic index of the cyclin D1−/− MEFs was assessed using annexin V immunohistochemistry in conjunction with propidium iodide staining as a sensitive measure of early apoptosis (53, 54). Cells that have become spontaneously permeable to PI indicate a breach in the membrane and are considered necrotic and were therefore excluded from analysis (53, 54). Scoring of annexin V immunostaining was performed in a double-blinded manner scoring at least 250 intact cells. The basal level of apoptosis was increased approximately 2-fold in the cyclin D1−/− as compared with cyclin D1+/+ MEFs from the same passage number (Fig. 7C). An increased basal rate of apoptosis in the cyclin D1−/− MEFs compared with wild type was observed in repeated experiments (data not shown). MEFs were plated on 0.5% gelatin, collagen monomer, and murine laminin to determine whether the matrix upon which the MEFs were plated independently affected the apoptosis rate (Fig. 7C). The
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Fig. 7. cyclin D1 and p300 inhibit apoptosis. A, JEG-3 cells were transfected with expression plasmids encoding cyclin D1, p300, or p300Δ1737−1836 together with pCMVEGFPspectrin. Transfected cells were identified by fluorescence-activated cell sorting. Cellular apoptosis was scored using propidium iodide-based FACS analysis to quantitate cells with subdiploid DNA content. Comparison is shown between the effect of the expression vector and its empty expression vector cassette (pCDNA3) as percent change in the sub-G1 population. B, apoptosis was assessed in MEFs by annexin V staining shown in green. Annexin V binds phosphotidylserine, and apoptotic changes result in increased concentrations of phosphotidylserine on the outer plasma membrane where it becomes accessible to annexin V (55). C, basal level apoptosis was measured by annexin V staining and PI co-staining in MEFs derived from wild type and cyclin D1−/− mice. Cells were plated on 0.5% gelatin, collagen (monomer), or laminin as indicated. A representative example from separate experiments is shown, in which more than 250 intact cells were scored in a double-blind manner for each cell-surface matrix.

rate of apoptosis was increased in the cyclin D1−/− MEFs compared with cyclin D1+/+ MEFs on each substrate (Fig. 7C). When plated on either collagen monomer or mouse laminin, the apoptotic rate was increased approximately 15–30% in the cyclin D1−/− MEFs. Together these studies suggest cyclin D1 and p300 can inhibit cellular apoptosis, and reduced cyclin D1 levels may be associated with enhanced basal apoptosis rates.

p300 Inhibits UV-induced Apoptosis in a Cyclin D1-dependent Manner—Our earlier experiments indicated that cyclin D1 and p300 could inhibit apoptosis and that the p300-responsive element of the cyclin D1 promoter bound predominantly c-Jun (Fig. 7C, lane 5). c-Jun protects from apoptosis induced by diverse stimuli including UV irradiation (72, 73), and embryonic fibroblasts from mice homozygozously deleted of the c-Jun gene (c-Jun−/− MEFs) display increased apoptosis rates (72).

Cyclin D1 is induced both by c-Jun and by apoptosis-inducing agents in several cell types (see Ref. 10 and reviewed in Ref. 74). We therefore examined the hypothesis that UV-induced apoptosis may be inhibited by cyclin D1. The functional interdependence of p300 and cyclin D1 in regulation of apoptosis was also assessed by comparing MEFs derived from cyclin D1−/− mice and their littermate wild type controls. Flow cytometry was performed in the presence of serum with or without UV irradiation (45 J/m2), to induce apoptosis.

UV irradiation (45 J/m2) increased the proportion of JEG-3 cells with subdiploid DNA content by 230% (Fig. 8A). The apoptosis induced by irradiation was reduced 30% by cyclin D1 overexpression (Fig. 8B). p300 overexpression reduced the amount of apoptosis by 25% (Fig. 8B). The CH3 deletion mutant, p300Δ1737−1836, failed to inhibit UV-induced apoptosis (Fig. 8B). These studies demonstrate that p300 inhibition of apoptosis requires the CH3 region, the same domain that was required for the induction of cyclin D1 expression.

Our studies raised the possibility that the induction of cyclin D1 may contribute to the p300-mediated inhibition of apoptosis. To examine this possibility, embryonic fibroblasts derived from either wild type mice or mice homozygozously deleted of the cyclin D1 gene were analyzed. To determine whether cyclin D1 abundance was critical for the ability of p300 to inhibit cellular apoptosis, comparison was made between the cyclin D1+/+ and cyclin D1−/− MEFs. Cells were transfected with the expression plasmids encoding p300 and pCMVEGFPspectrin. Apoptosis was scored using FACS analysis to quantitate apoptotic cells with subdiploid DNA content. Comparison was made with cells transfected with equal amounts of empty expression vector cassette (pCDNA3). p300 overexpression inhibited apoptosis 25% in the wild type fibroblasts but did not inhibit apoptosis in the cyclin D1−/− fibroblasts (Fig. 8C). Indeed p300 increased the proportion of sub-G1 cells by 25% in the cyclin D1−/− fibroblasts. These studies suggest that cyclin D1 inhibits cellular apoptosis, and the abundance of cyclin D1 determines the ability of p300 to inhibit apoptosis.

**DISCUSSION**

The p300 gene encodes a co-activator protein with partial functional redundancy based on genetic analysis. The orthologue CBP and co-activator proteins of the p160 SRC family contribute co-activator function and target overlapping genetic and transcription factor targets. The molecular mechanisms by which p300 regulates specific components of the cell cycle remained to be determined. As p300 can inhibit cellular proliferation in a cell type-specific manner, analysis of the interface between co-activators and specific target genes will be complex. In the current study, p300 induced cyclin D1; however, CBP, although capable of inducing RAR-reporter activity, failed to induce cyclin D1. This finding is consistent with recent studies in which p300 and CBP induced distinct transcriptional targets in the same cell type (41). CBP induced p21^Cip1 and p300 induced p21^Cip1 in F9 cells (41). The findings that p300 induced cyclin D1 through DNA sequences that bind c-Jun/c-Fos together with p300, rather than other candidate transcription factor binding sites in the promoter, indicate specificity in the signal transduction pathways by which p300 regulates a specific target gene. p300, together with c-Jun and c-Fos, formed part of the cyclin D1 promoter DNA-bound nuclear protein complex at −953. p300 augments the activity of c-Jun and...
The apoptosis rates after UV irradiation were increased up to 100% in the cyclin D1 knockout MEFs compared with wild type MEFs on several different substrates (type 1 collagen monomer, laminin, and 0.5% gelatin). Cyclin D1 is induced both by c-Jun directly (10) and in a JNK-dependent manner in response to oncogenic pp60src (18). Future studies will determine the role for cyclin D1 overexpression in resistance to apoptosis induced by other agents, such as chemotherapeutics; however, the inhibition of apoptosis by cyclin D1 may contribute to aberrant survival and oncogene-induced tumorigenesis.

The CH3 region of p300 can play a role in recruitment of P/CAF and its associated histone acetylase activity. Acetylation of histones is associated with gene activation, and several transcription co-activators/adaptors convey histone acetyltransferase activity, including Gcn5 family members (hGcn5, P/CAF), CBP/p300, and TAFII250 (79). Several lines of evidence support a role for histone acetyltransferase activity conveyed by these proteins in cell cycle regulatory events. P/CAF overexpression, however, did not induce cyclin D1 in JEG-3 cells (data not shown), suggesting the formation of distinct multimeric complexes at the CH3 domain may convey separate functional activities. Because P/CAF can inhibit cell cycle progression (80), the ability of p300 to recruit distinct complexes to a common region may be critical in determining its cell cycle effects. Although CBP did not induce cyclin D1, consistent with the ability of p300 and CBP to regulate distinct target genes through distinct transcription factors (40, 75), other co-activators may enhance cyclin D1 abundance in other cell types. For example cyclin D1 promoter activity is induced by activity of the TAFII250 subunit of the basal transcription complex TFIID (81). Cells expressing mutants of TAFII250 are deficient in their rate of G1 phase progression, and cells expressing Gcn5 mutants defective in histone acetyltransferase activity have a proliferative defect (82).

Efficient transformation by E1A involves cooperative functions between early adenoviral genes that inhibit apoptosis and stimulate cell cycle progression. The differential regulation by EIA12S of cyclin D1 compared with cyclin A likely reflects distinct cell cycle regulatory mechanisms. In the current studies EIA12S inhibition of both cyclin D1 and cyclin E required the amino-terminal p300 binding domain. Cyclin A was activated by E1A12S and was unaffected by mutation of the E1A amino terminus. Previous studies in cultured cells had shown that E1A inhibited cyclin D1 mRNA and protein (29, 37, 38) and that activation of cyclin A by E1A12S involved a p107-dependent mechanism (69). Our studies are consistent with a role for cyclin D1 as a downstream gene targeted for inhibition by E1A12S as part of the adenoviral anti-apoptotic function. Several observations in the current study are consistent with a role for cyclin D1 as an inhibitor of apoptosis. UV irradiation-induced apoptosis was inhibited by overexpression of either p300 or cyclin D1. Embryonic fibroblasts derived from mice homozygously deleted of the p300 gene showed increased apoptosis compared with MEFs of identical passage number from wild type fibroblasts. p300 also inhibited apoptosis in UV-irradiated wild type MEFs. In contrast, p300 overexpression did not inhibit apoptosis in the cyclin D1 knockout MEFs, in fact there was a modest but reproducible induction. These findings are consistent with the finding of increased retinal apoptosis in the cyclin D1 knockout retina (24) and in contrast with studies in which cyclin D1 inductively overexpressed in cultured neural (83) or mammary epithelial cell (26) increased apoptosis rate. Although the mechanisms governing apoptosis by cyclin D1 are likely complex and cell type-specific, these studies provide important insights into the role of basal cyclin D1 abundance in apoptosis and suggest an important role for cyclin D1 in p300-
mediated inhibition of apoptosis.

The inhibition of apoptosis by cyclin D1 may contribute to its transforming function in specific cell types. E1A cooperates with cyclin D1 in cellular transformation (84), and E1A can inactivate cellular reponses needed for Ras-mediated inhibition of cellular growth by converting ras into a growth-promoting gene (85). Expression of E1A is insufficient to transform baby rat kidney cells because cell cycle disruption by E1A also stimulates p53-dependent apoptosis (86). Both Myc and E1A are potent inducers of apoptosis (52, 61, 87) and share the ability to inhibit cyclin D1-mediated apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36). Both Myc and E1A are potent inducers of apoptosis (36).
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