The Inhibitor of Cyclin-Dependent Kinase 4a/Alternative Reading Frame (INK4a/ARF) Locus Encoded Proteins p16INK4a and p19ARF Repress Cyclin D1 Transcription through Distinct cis Elements

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ABSTRACT

The Ink4a/Arf locus encodes two structurally unrelated tumor suppressor proteins, p16INK4a and p14ARF (murine p19ARF). Invariant inactivation of either the p16INK4a-cyclin D/CDK-pRb pathway and/or p53-p14ARF pathway occurs in most human tumors. Cyclin D1 is frequently overexpressed in breast cancer cells contributing an alternate mechanism inactivating the p16INK4a-pRb pathway. Targeted overexpression of cyclin D1 to the mammary gland is sufficient for tumorigenesis, and cyclin D1−/− mice are resistant to Ras-induced mammary tumors. Recent studies suggest cyclin D1 and p16INK4a expression are reciprocal in human breast cancers. Herein, reciprocal regulation of cyclin D1 and p16INK4a was observed in tissues of mice mutant for the Ink4a/Arf locus. p16INK4a and p19ARF inhibited DNA synthesis in MCF7 cells. p16INK4a repressed cyclin D1 expression and transcription. Repression of cyclin D1 by p16INK4a occurred independently of the p16INK4a-cdk4-binding function and required a cAMP-response element/activating transcription factor-2-binding site. p19ARF repressed cyclin D1 through a novel distal cis-element at −1137, which bound p53 in chromatin-immunoprecipitation assays. Transcriptional repression of the cyclin D1 gene through distinct DNA sequences may contribute to the tumor suppressor function of the Ink4a/Arf locus.

INTRODUCTION

The cyclin D1 gene encodes a labile regulatory subunit of a holoenzyme that phosphorylates and inactivates the pRb retinoblastoma tumor suppressor. Oncogenic signals including Ras, Src, Stat3, and β-catenin induce the transcription and abundance of cyclin D1 (1–4). Cyclin D1 is overexpressed in more than 30% of human breast cancers and mammary gland-targeted expression of cyclin D1 is sufficient for the induction of mammary tumorigenesis (5). Conversely, the reduction in cyclin D1 abundance using antisense and immunoneutralizing antibodies has demonstrated a requirement for cyclin D1 in proliferative signaling induced by growth factors and hormones in breast cancer epithelial cells (6, 7). Mice deleted of the cyclin D1 gene are resistant to Ras- and ErbB2-induced mammary tumorigenesis (8). Antiproliferative agents such as endostatin (9) and pharmaceutical agents with antiproliferative properties including acyclic retinoid, flavoperidol, and phosphatidylinositol 3′-kinase inhibitors repress cyclin D1 transcription and abundance leading to cell cycle arrest (10–12). The tumor suppressors RASSF1A and the INI1 repressor components of the SWI/SNF complex mediate cell cycle arrest through repression of cyclin D1 (13, 14). As a common transcriptional target of diverse oncogenic and mitogenic signaling pathways through distinct DNA sequences, cyclin D1 transcription provides the basis for collaborative induction by proliferative pathways (15). Conversely, transcriptional repression of cyclin D1 by cytosolic molecules serves to integrate in a dynamic manner cell cycle inhibitor effectors.

The complex interplay between tumor-suppressor genes and tumor-promoting genes has been simplified through the almost invariant inactivation of the p53 and the p16INK4a/retinoblastoma (Rb) pathway in human cancer (16, 17). Cyclin D1 overexpression provides a common mechanism for disruption of the Rb pathway in human breast cancers. In other tumor types, Rb pathway inactivation may occur as a result of inactivation of Rb through mutation, deletion, or inactivation of Rb function through viral sequestration, phosphorylation, or dysregulation of components controlling Rb phosphorylation (18). Mutations of G1-specific cyclin-dependent kinase catalytic units and/or elimination of inhibitor(s) of cyclin-dependent kinase 4 (INK4) also contribute to the phosphorylation status and inactivation of Rb (16, 17). The Ink4a/Arf locus is involved in both the pRb and p53 pathways encoding p16INK4a, a regulator of cdk4/6-mediated pRB phosphorylation, and p19ARF, a modulator of Mdm2-mediated degradation of p53 (19, 20). Mice deleted of the Ink4a/Arf locus are prone to spontaneous tumor formation and have increased susceptibility to oncogenic effects of Ras, Myc, and an activated epidermal growth factor receptor (21–23) indicating the importance of this locus in tumor suppression in vivo.

Activating signals from β-catenin, Src, and the Ras-Raf-MAPK pathway induce Ink4a/Arf (24–27). Thus common oncogenic signals induce expression of both cyclin and cyclin inhibitors. The fidelity with which Ink4a/Arf, induced by oncogenic signals, transduces a tumor suppressor phenotype is of fundamental importance (16, 17). Ectopic p19 alternative reading frame (ARF) expression stabilizes p53 and induces p53-responsive genes, including the p21cip1 gene that contributes to cell cycle arrest in fibroblasts (28). The molecular targets of Ink4a/Arf in mammary epithelial cells are not well understood, although loss of p16INK4a in breast cancer cells contributes to extended growth capacity (29), and reintroduction of p16INK4a into breast cancer epithelial cells induces a dose-dependent cell cycle arrest (30, 31). Breast cancer cell lines are deleted of p16INK4a in 40–60% of cases (32), and DNA methylation, an alternate mechanism of p16INK4a inactivation, has been reported to occur in up to 30% of human breast cancers (33, 34). Reciprocal expression of cyclin D1 and p16INK4a has been reported in human breast cancer (34), raising the possibility that p16INK4a may regulate cyclin D1 abundance. The current studies investigated the function of Ink4a/Arf in mammary epithelial cells and the mechanism of cell cycle arrest. The expression of cyclin D1 is increased in the tissues of Ink4a/Arf−/− mice, and cyclin D1 is a target of transcriptional repression by INK4a and
ARF through distinct DNA sequences in the promoter. As cyclin D1 abundance is rate-limiting in breast epithelial cell proliferation and tumorigenesis, INK4a and ARF repression of cyclin D1 may contribute to INK4a/ARF tumor suppression function.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reporter Gene Assays.** The luciferase reporter gene constructions for human cyclin D1, cyclin E, cyclin A, c-Fos, c-Jun, JunB, p16INK4a (6, 35), and expression vectors for p16INK4a, pCMV-p16INK4a, pCMV-p16INK4aP114L (36) and pcDNA-p19ARF were described previously. The region from -1156 to -1117 was deleted in the context of the -1745 CD1LUC reporter.

Cell culture, DNA transfection, and luciferase assays were performed as described previously (35). The MCF7 cells were maintained in MEM with 5% FCS and 1% penicillin/streptomycin. Cells were transfected by calcium phosphate precipitation, the media was changed after 3 h, and luciferase activity determined after a further 24–36 h. Comparison was made between the effect of transfecting active expression vector with the effect of an equal amount of vector cassette. At least three different plasmid preparations of each construct were used. In each experiment, a dose response was determined with 150, 300, or 600 ng of expression vector as indicated in the figure legend (3,4,6,8) and the cyclin D1 promoter reporter plasmids (4.8 μg). Luciferase assays were performed at room temperature using an Autolumat LB 953 (EG&G Berthold), and the initial 30 s of the reaction were used to assess luciferase content with the values expressed in arbitrary light units. Background activity from cell extracts was typically 100 Absolute Light Units/10 s. Statistical analyses was performed using the Mann-Whitney U test, and significant differences were established as P < 0.05.

**Antibodies, Reagents, and Northern and Western Blot Analysis.** MCF7 cells were transfected with empty vector or expression plasmids encoding p16INK4a or p19ARF along with pMACS4.1 and sorted for transfection using an autoMACS cell sorter (Miltenyi Biotech, Auburn, CA). Cellular extracts were then processed for either Northern or Western blot analysis as described-deficient previously in this laboratory (11, 35). Tissues isolated from Ink4a/Arf-deficient mice, and cells were lysed in buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 mg/ml leupeptin, and 0.1 mM Na3VO4. Total protein was determined using the Bradford method (Bio-Rad, Hercules, CA). Lysates (100 μg) were separated on 12% SDS-PAGE and blotted onto nitrocellulose. Membranes were blocked with 5% milk in PBS-0.2% Tween 20 for 1 h at room temperature before exposure of primary antibodies to cyclin D1 (AB-3; NeoMarkers, Fremont, CA), p16INK4a, p19ARF, p53, activating transcription factor-2 (ATF-2), cAMP-responsive element-binding protein (CREB; Santa Cruz Biotechnology, Santa Cruz, CA), or guanine dissociation inhibitor (a gift from Dr. P. Bickel). Membranes were
fold change is shown, compared with the effect of equal amounts of empty expression vector cassette for the effect of the p16 INK4a expression vector. Northern blot analysis of MCF7 cells transfected with p16^{INK4a}, p19^{ARF} expression plasmid, and cells transfected with empty vector cassette. Cells were stained with cell-permeable DNA-binding dye Hoechst 33342 (10 mg/ml) for 2 h before harvesting, and all experiments were performed following a protocol provided by Upstate Biotechnology under modified conditions. MCF7 cells (1 × 10^6) were grown in DMEM with 10% FBS-0.2% Tween 20, and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Membranes were washed extensively with PBS-0.2% Tween 20, and proteins were visualized using chemiluminescence. Fold increase in protein levels were plotted against a standard of 293T cells transfected with the expression vector for p16^{INK4a} wild type or the cdk-binding-defective p16^{INK4a} mutant. The data are mean ± SEM of N = 8 separate experiments.

Flow Cytometric Analyses. Selection of transfected cells using the CD4 as a marker was performed as described previously using magnetic activated cell sorting (MACS; Ref. 37). Flow cytometric analyses were carried out in a fluorescence-activated cell sorter (FACStar plus; Becton Dickinson) with a 360–365 nm argon-iron laser (38). Histograms were analyzed for cell cycle compartments using ModFit version 2.0 (Verity Software House, Topsham, ME). To select transfected cells, cotransfection experiments were conducted using magnetic separation of transfected cells using CD4 as the marker and the MACS. A comparison was made between cells transfected with vector encoding p16^{INK4a}, p19^{ARF} expression plasmid, and cells transfected with empty expression vector cassette. Cells were stained with cell-permeable DNA-binding dye Hoechst 33342 (10 mg/ml) for 2 h before harvesting, and all experiments were performed following a protocol provided by Upstate Biotechnology under modified conditions. MCF7 cells (1 × 10^6) were grown in DMEM with 10% FBS-0.2% Tween 20, and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Membranes were washed extensively with PBS-0.2% Tween 20, and proteins were visualized using chemiluminescence. Fold increase in protein levels were plotted against a standard of 293T cells transfected with the expression vector for p16^{INK4a} wild type or the cdk-binding-defective p16^{INK4a} mutant. The data are mean ± SEM of N = 8 separate experiments.

Fig. 3. The cyclin D1 promoter is repressed by p16^{INK4a} in MCF7 breast epithelial cells. A and B, luciferase activity of the cyclin D1 promoter (−1745 CD1LUC; 4.8 μg) and either viral, immediate early gene, or cell cycle control promoters were determined in MCF7 cells transfected with expression vectors for p16^{INK4a} (control, □), 150, □; 300, □; and 600 ng, □). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; INK, inhibitor of cyclin-dependent kinase; LUC, luciferase. Inset, the data are shown as mean ± SE for N > 8 separate experiments. Fold change is shown, compared with the effect of equal amounts of empty expression vector cassette for the effect of the p16^{INK4a} expression vector. Northern blot analysis of MCF7 cells transfected with p16^{INK4a} and subjected to magnetic activated cell sorting of transfected cells. C, comparison of cyclin D1 promoter repression by either p16^{INK4a} wild type or the cdk-binding-defective p16^{INK4a} mutant. The data are mean ± SEM of N = 8 separate experiments.

Ink4a/Arf^{−/−} Mice. The Ink4a/Arf^{−/−} knockout mice, with portions of exons 2 and 3 replaced by a neo cassette (40), were established in the Friends virus C strain through 11 back-crossings. Mice were screened by PCR for the genomic status. DNA was extracted by standard methods from a small piece of tail tissue cut from each animal at the time of weaning (41). For evaluating the Ink4a/Arf status of offspring, PCR reactions using three primers allowed for simultaneous detection of both the normal and mutant p16^{INK4a} allele in a single reaction. These primers consisted of a common 5′ sense primer, p16−1F (5′TCCCTCTACTTTCTTCTGAC-3′); two 3′ antisense primers, p16−1R (5′CGGAAGCCCAAATATCGCAC-3′); and p16−Nul (5′CTAGTGAGACGTGCTACTTC-3′). Reactions were run for 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Mouse embryo fibroblasts were prepared from Ink4a/Arf^{−/−} or wild-type litter mate controls as described previously (11).

Chromatin Immunoprecipitation (ChIP) Assay. ChIP analysis was performed following a protocol provided by Upstate Biotechnology under modified conditions. MCF7 cells (1 × 10^6) were grown in DMEM with 10% charcoal stripped dextran serum for 3 days. Upon estradiol (100 nm) stimulation for 45 min, the cells were cross-linked by adding 1.1% formaldehyde buffer containing 100 nm sodium chloride, 1 mM EDTA-Na, 0.5 mM EGTA-Na, and Tris-HCl (pH 8.0) directly to culture medium for 10 min at 365 nM argon-iron laser. Western blotting was performed on the cells as described above (39).

Fig. 4. Repression of the cyclin D1 promoter by p16^{INK4a}. Luciferase activity of the cyclin D1 promoter (−1745 CD1LUC) in the presence or absence of p16^{INK4a} (control, □), 150, □; 300, □; and 600 ng, □). INK, inhibitor of cyclin-dependent kinase; CMV, cytomegalovirus; LUC, luciferase; HEK, human embryonic kidney.
Fig. 5. The cyclin D1 promoter ATF/CRE site is required for p16\(^{\text{INK4a}}\) repression in MCF7 cells. A, luciferase activity from either the wild type (wt; \(-1745\)CD1LUC, 4.8 \(\mu\)g) or the cyclin D1 promoter luciferase reporters with point mutations of the TCE or CRE site was compared for repression by p16\(^{\text{INK4a}}\) (300 ng) in either MCF7 or (B) SKBR3 cells. The data are shown as mean \pm SEM for \(N > 8\) separate experiments. INK, inhibitor of cyclin-dependent kinase; LUC, luciferase; TCE, T cell factor; CRE, relative luciferase activity of the \(-1745\)CD1LUC (4.8 \(\mu\)g) was compared in cells cotransfected with either cAMP-responsive element-binding protein or ATF-2 wt or dominant-negative mutant expression vector using either 300 (\(\square\)) or 600 ng (\(\boxplus\)). Comparison was made between the effect of the wt or dominant-negative vector and equal amounts of empty expression vector cassette. MCF7 cells transfected and magnetic activated cell sorted with the empty control vector and expression vector for p19\(^{ARF}\). Western blot of transfected cells are shown. Antibodies were used as shown. ATF, activating transcription factor; CRE, cAMP-response element; ARF, alternative reading frame; GDI, guanine dissociation inhibitor; DN, dominant-negative; DBD LZ, DNA-binding domain leucine zipper.

37°C. The medium was aspirated, washed thrice using ice-cold PBS containing 10 mM DTT and protease inhibitors, lysed by warm 1% SDS lysis buffer, and incubated for 10 min on ice. The cell lysates were sonicated to shear DNA to lengths between 200 and 1000 bp, and the samples were diluted to 10-fold in ChIP dilution buffer. To reduce nonspecific background, cell pellet suspension was precleared with 60 \(\mu\)l of salmon sperm DNA/protein-A agarose-50% slurry (Upstate Biotechnology) for 2 h at 4°C with agitation. Chromatin solutions were precipitated overnight at 4°C using 4 \(\mu\)g of anti-p53 (FL-393; Santa Cruz Biotechnology) with rotation. For a negative control, rabbit IgG was immunoprecipitated by incubating the supernatant fraction for 1 h at 4°C with rotation. Sixty \(\mu\)l of salmon sperm DNA/protein-A agarose slurry was added for 2 h at 4°C with rotation to collect the antibody/histone complex and washed extensively following the manufacturer’s protocol. Input and immunoprecipitated chromatin were incubated at 65°C overnight to reverse crosslinks. After protease K digestion for 1 h, DNA was extracted using Qiagen spin column kit. Precipitated DNAs were analyzed by PCR. ChIP analysis was conducted of either endogenous gene promoters or in a subset of experiments, MCF-7 cells were transiently transfected with plasmid DNA encoding promoter reporter gene constructs, and ChIP analysis was conducted using a reduction in the number of cycles from 30 to 25. The following primers were used for PCR: for mdm2 promoter, 5’-TGGGCCAGGTTGACTCAGCCTTCTCCTC-3’ and 5’-TGGCGTGCGTCCGTGCCCAC-3'; primer pair for ARF-response element site, 5’-GCCCCCTCCGCTCCTACCT-3’ and 5’-TGGGGCTCTTCCTGGGCAGC-3’. MCF7 cells were transiently transfected with either cyclin D1 \(-1745\) or cyclin D1 \(-1745\)-ARF-response element deletion mutant expression vector followed by treatment with estradiol for 45 min. The cells were cross-linked with formaldehyde buffer for 10 min at 37°C and followed the procedure as mentioned above.

RESULTS

Increased Cyclin D1 Abundance in Ink4a/Arf Deficient Cells. The p16\(^{\text{INK4a}}\) tumor suppressor was initially defined through its ability to inhibit Ras-induced transformation (42) and embryonic fibroblasts from Ink4a/Arf \(-/-\) mice [mouse embryonic fibroblast (MEFs)] exhibit enhanced cellular proliferation. Conversely, cyclin D1-deficient mice are resistant to Ras-induced transformation and cyclin D1 \(-/-\) MEFs show reduced cellular proliferation (42). The transcriptional targets of p16\(^{\text{INK4a}}\) mediating tumor suppressor func-
tion and inhibition of DNA synthesis remain to be fully defined. Oncogenic Ras was previously shown to induce cyclin D1 in rodent fibroblasts and human trophoblast cells (2). We investigated the possibility that cyclin D1 may function as a Ras-inducible gene that is a target of p16INK4a repression.

Consistent with previous studies, the proliferation rates of Ink4a/Arf−/− MEFs were increased compared with litter mate controls (Fig. 1A) and Ink4a/Arf+/− MEF proliferation rate was greater than Ink4a/Arf−/− MEFs (Fig. 1B). To determine whether reduced p16INK4a levels were associated with increased abundance of cyclin D1 in vivo, we examined the tissues of mice genetically deficient for Ink4a/Arf−/− (40). (These mice were generated by deleting exons 2 and 3 of the Cdkn2a locus and are functional knockouts for both p16INK4a and p19ARF genes, which share exons 2 and 3 of the Cdkn2a locus). Compared with the Ink4a/Arf−/− mice, the Ink4a/Arf-deficient mice tissues showed 2- to 3-fold increased abundance of cyclin D1 protein (Fig. 1C). The cytoplasmic protein, guanine dissociation inhibitor, used as a loading control, showed similar abundance of protein between genotypes.

**p16INK4a and p19ARF Inhibits DNA Synthesis and Apoptosis in MCF7 Breast Cancer Epithelial Cells.** To investigate the mechanisms by which p16INK4a or p19ARF regulate DNA synthesis, MCF7 cells were transfected with expression vectors for either p16INK4a or p19ARF and subjected to MACS sorting with subsequent fluorescence-activated cell sorter analysis and Western blotting. p16INK4a reduced S-phase by 40% (Fig. 2), consistent with previous studies (31), and p19ARF reduced the S-phase fraction by 30%, consistent with previous studies in fibroblasts (43).

**Selective Repression of Cyclin D1 Promoter by p16INK4a.** Northern blot analysis of transfected and MACS-sorted MCF7 cells showed that p16INK4a reduced cyclin D1 mRNA (Fig. 3A, right). p16INK4a repressed the human cyclin D1 promoter, in a dose-dependent manner (Fig. 3A) and reduced cyclin D1 protein levels 40% (data not shown). To assess further the mechanism of cyclin D1 promoter transcriptional repression by p16INK4a, the promoters of several cell cycle control genes were assessed. p16INK4a did not inhibit the activity of the human c-fos promoter, consistent with a previous study in which p16INK4a failed to repress the serum-response element of the c-fos promoter (42). The PAULUC vector reporter was unaffected by p16INK4a, indicating that the effect was not mediated by a general effect on luciferase activity (Fig. 3A). The viral cytomegalovirus (CMV) promoter was induced 3-fold by p16INK4a.

The cyclin E promoter was repressed by p16INK4a (Fig. 3A); however, the cyclin A promoter was unaffected, and the p21CIP1/WAF1 promoter was induced; thus p16INK4a conveys promoter-specific regulatory effects. The p16INK4a mutant defective in binding cdk4 (p16INK4a-P114L; Ref. 36) repressed basal cyclin D1 promoter activity to a similar degree as p16INK4a wild type (Fig. 3B). Thus the mechanism by which p16INK4a repression basal activity of the cyclin D1 promoter appears to be independent of its cdk4-binding function. Because p16INK4a failed to repress cyclin A, which is induced during S-phase entry, and the cdk4-binding-defective mutant repressed the cyclin D1 promoter, these findings raise the possibility that the effect of p16INK4a on cyclin D1 promoter activity is not mediated indirectly through effects on DNA synthesis.

Cyclin D1 abundance regulates MCF7 cell cycle progression, making a determination of the effect of p16INK4a independent of cell cycle complex. The ability of cyclin D1 to induce cell cycle progression is dependent upon the presence of the pRB protein, which is phosphorylated and inactivated by cyclin D1 kinase activity (44, 45). pRB is inactivated by E1A and large T antigen (46). We therefore assessed the effect of p16INK4a on transcriptional regulation of the cyclin D1 promoter.
promoter in E1A and large T antigen transformed 293T cells. Cyclin D1 promoter activity was repressed by p16INK4a (Fig. 4A), while inducing the activity of the c-fos, c-jun, cdc25a, and p21Cip1/WAF1 promoters. In contrast the CMV promoter was unaffected, and the cyclin E promoter was repressed (Fig. 4A and B).

**p16INK4a Repression of the Cyclin D1 Promoter Requires the CRE/ATF-2-Binding Site in MCF7 Breast Epithelial Cells.** Expression of cyclin D1 is induced by diverse signaling pathways in MCF7 cells including Ras, Wnt, and ErbB2 through distinct DNA sequences including a T cell factor (Tcf), E2F, and ATF/CRE site. Point mutation of the ATF/CRE site, but not the Tcf site, abrogated repression and resulted in a promoter fragment that was activated by p16INK4a (Fig. 5A). Mutation of the ATF/CRE site also abolished repression by p16INK4a in the human SKBR3 breast cancer cell line (Fig. 5B). As the CRE site of the cyclin D1 promoter binds CREB and ATF-2 protein in epithelial cells (35, 47), we assessed the role of these proteins in regulating cyclin D1 promoter activity in MCF7 cells. ATF-2 inhibited cyclin D1 promoter activity 35–40%. In contrast, a DNA-binding-defective mutant of ATF-2 that dimerizes with AP-1 proteins and functions as a dominant-negative mutant of ATF-2 function, induced cyclin D1 (Fig. 5C). CREB induced and a dominant-negative CREB mutant inhibited cyclin D1 promoter activity (Fig. 5C). MCF7 cells transfected with p16INK4a and MACS sorted reduced ATF-2 and CREB protein 40–50%. Together these studies indicate that CREB/ATF-2 protein expression regulates basal cyclin D1 abundance in MCF7 cells, and p16INK4a repression of cyclin D1 involves the ATF-2/CRE site in the cyclin D1 promoter in breast cancer epithelial cells.

**Repression of Cyclin D1 by p19ARF Functions through a Novel Distal Response Element.** The reciprocal regulation of cyclin D1 and p16INK4a abundance has been observed in human breast cancers (34, 47) and in the Ink4a/Arf−/− mice mammary gland (48, 49). The Ink4a/Arf−/− mice are deficient in both p16INK4a and p19ARF protein. We therefore examined the role of p19ARF in regulating cyclin D1 promoter activity in MCF7 cells. p19ARF repressed cyclin D1, the cyclin A and p21Cip1 promoters in a dose-dependent manner, without affecting the activity of the viral CMV-LUC reporter (Fig. 6A). Mutation of the Tcf site or the AP-1 and CRE site also did not reduce repression but rather enhanced repression by p19ARF, p19ARF transfection of MCF7 cells induced p53 and reduced cyclin D1 protein levels consistent with previous studies that p19ARF functions in a genetic pathway that involves p53 (Refs. 49, 50; data not shown). Mutation of the ATF/CRE site that had abolished repression by p16INK4a did not affect repression by p19ARF (Fig. 6B). p53 is capable of regulating gene expression either directly through consensus p53-response elements or through atypical response elements upon physical interaction with other proteins including WT1-Egr and nuclear receptors (51–53). We scrutinized the sequences of the cyclin D1 promoter for DNA sequences that could potentially respond to p53 either directly or indirectly. In the absence of a consensus p53-response element, we mutated elements resembling an atypical p53-response element in the cyclin D1 promoter at −1137 (54), p19ARF-mediated repression was abrogated by deletion of the sequences, strongly suggesting a key role for this element in p19ARF repression of cyclin D1 transcription (Fig. 6C).

To further examine the role of p53 in the repression of the cyclin D1 promoter by p19ARF, we performed ChIP assays in MCF7 cells. Oligonucleotides were generated for PCR amplification of the p53-response element of the mdm2 promoter as a form of positive control for the ChIP assays and to the cyclin D1 promoter CRE site, E2F site, and the ARF-response element. p53 bound the mdm2 promoter p53-response element in ChIP assays but was not identified at the cyclin D1 promoter CRE site. In contrast, p53 was contained within ChIP assays of the cyclin D1 promoter ARF-response element and E2F site (Fig. 7A). Transfected cyclin D1 promoter recruited p53 to the ARF-response element but did not bind p53 at the CRE site. Mutation of the cyclin D1 promoter ARF-response element abrogated p53-binding to the promoter in ChIP assays, strongly suggesting this site is required for p53 binding to the cyclin D1 promoter (Fig. 7).

To determine whether p19ARF repression of cyclin D1 transcription was unique to MCF7 cells, we assessed transcriptional regulation in the embryonal kidney cell line, 293T. **Cyclin D1 and cyclin A promoter activity was repressed in a dose-dependent manner by p19ARF, without affecting CMV promoter activity (Fig. 8A).** There was a modest induction of the c-Jun promoter (Fig. 8B), p19ARF repressed the p16INK4a promoter (Fig. 8C) consistent with previous findings that expression of p16INK4a is high in ARF-null keratinocytes and bone marrow macrophages (55, 56). 293T cells express E1A and large T, functionally inactivating the pRb and p53-response element. Consistent with other studies of ARF target genes, these findings suggest ARF repression of the cyclin D1 promoter may involve both p53-dependent and p53-independent signaling mechanisms. Together these studies demonstrate that the cyclin D1 gene is repressed by both reading frames transcribed from the INK4/ARF locus, and transcriptional repression is mediated through two distinct DNA sequences (Fig. 8D).

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**Fig. 7. ChIP assays reveal p53 is bound to the cyclin D1 promoter.** A. MCF7 cells were subjected to chromatin immunoprecipitation assays. PCR using oligonucleotides flanking the cyclin D1 ARF-response element (ARE), and E2F site (E2F) revealed a band in samples immunoprecipitated with antibodies to p53 corresponding to the endogenous cyclin D1 promoter. CRE. cAMP-response element; LUC, luciferase. B. MCF7 cells transfected with a cyclin D1 promoter construct also indicated that p53 was bound to the ARF-response element (ARE) and E2F sites. C. p53 was not bound in MCF7 cells transfected with a mutant cyclin D1 promoter construct.
In Ink4a/Arf-deficient tissues, and both p16 INK4a and p19 ARF inhibited cyclin D1 abundance. Increased cyclin D1 protein abundance was observed in several transcriptional control by both components of the INK4a/ARF locus. Increased cyclin D1 levels in mammary tumors of transgenic mice induced by ErbB2 that were Ink4a/Arf-deficient (49). The findings of increased cyclin D1 levels in mammary tumors of transgenic mice induced by ErbB2 that were Ink4a/Arf-deficient (49). The INK4a/ARF -encoding p16 INK4a repressed cyclin D1 in E1A and large T overexpressing tumors. First, p16INK4a displayed promoter-selective function and was capable of either repressing (cyclin D1, Jun B) or activating (c-jun, cyclin A) transcription of specific target genes. Second, repression by p16INK4a displayed sequence-selective function. Third, a cdk4-binding-defective mutant of p16INK4a repressed the cyclin D1 promoter. Fourth, p16INK4a repressed cyclin D1 in E1A and large T overexpressing 293T cells. As pRb is inactivated by E1A and large T antigen (46), an indirect effect of p16INK4a on cyclin D1 through the pRb protein (44, 45) seems less likely. Fifth, the cyclin D1 gene is regulated by pRb and E2F/DP proteins through an E2F binding site (6, 62). Studies with dominant-negative E2F mutants demonstrated that the cyclin D1 E2F sites are functional in MCF7 cells (6). The p16INK4a-induced cell cycle arrest that correlates with a reduction in pRb phosphorylation corresponds to a repression of E2F-regulated genes (36, 63, 64). In the current studies, the E2F-responsive elements of the cyclin D1 promoter were not involved in the transcriptional repression by p16INK4a and p19ARF. Together these findings suggest the mechanism by which p16INK4a represses cyclin D1 transcription described herein is distinct from previous studies in which p16INK4a regulated expression of cell cycle control genes indirectly through binding cdk4 and inhibiting cyclin D1 kinase activity.

**DISCUSSION**

The current studies provide evidence that the cyclin D1 gene is under transcriptional control by both components of the INK4a/ARF locus. Increased cyclin D1 protein abundance was observed in several Ink4a/Arf-deficient tissues, and both p16INK4a and p19ARF inhibited cyclin D1 abundance in MCF7 cells. The increased abundance of cyclin D1 in Ink4a/Arf-deficient tissues is consistent with recent findings of increased cyclin D1 levels in mammary tumors of transgenic mice induced by ErbB2 that were Ink4a/Arf-deficient (49). The Ink4a/Arf deletions (23, 57, 58), like cyclin D1 overexpression, correlate with poor outcome from tumors in patients and in mice. As the abundance of cyclin D1 is rate limiting in G1 phase progression in mammary epithelial cells (7), regulation of cyclin D1 abundance by p16INK4a and p19ARF may contribute to the cell cycle control or tumor suppressor functions of these proteins.

Although p16INK4a was originally identified as a protein binding to cdk4 that acts upstream of Rb to cause G1 arrest (59), cdk4-binding independent functions of p16INK4a contribute to cell cycle arrest (60, 61). Several lines of evidence suggest the mechanism of transcriptional repression of the cyclin D1 promoter by p16INK4a is specific and not an indirect effect of the expressed proteins on cell cycle control. First, p16INK4a displayed promoter-selective function and was capable of either repressing (cyclin D1, Jun B) or activating (c-jun, cyclin A) transcription of specific target genes. Second, repression by p16INK4a displayed sequence-selective function. Third, a cdk4-binding-defective mutant of p16INK4a repressed the cyclin D1 promoter. Fourth, p16INK4a repressed cyclin D1 in E1A and large T overexpressing 293T cells. As pRb is inactivated by E1A and large T antigen (46), an indirect effect of p16INK4a on cyclin D1 through the pRb protein (44, 45) seems less likely. Fifth, the cyclin D1 gene is regulated by pRb and E2F/DP proteins through an E2F binding site (6, 62). Studies with dominant-negative E2F mutants demonstrated that the cyclin D1 E2F sites are functional in MCF7 cells (6). The p16INK4a-induced cell cycle arrest that correlates with a reduction in pRb phosphorylation corresponds to a repression of E2F-regulated genes (36, 63, 64). In the current studies, the E2F-responsive elements of the cyclin D1 promoter were not involved in the transcriptional repression by p16INK4a and p19ARF. Together these findings suggest the mechanism by which p16INK4a represses cyclin D1 transcription described herein is distinct from previous studies in which p16INK4a regulated expression of cell cycle control genes indirectly through binding cdk4 and inhibiting cyclin D1 kinase activity.

p16INK4a and p19ARF inhibited cyclin D1 promoter activity through distinct DNA sequences. The CRE/ATF-2 site of the cyclin D1 promoter was required for repression by p16INK4a in MCF7 cells and SKBR3 cells. In our previous studies, electrophoretic mobility shift assays identified CREB and ATF-2 as the dominant proteins binding the cyclin D1 CRE site in MCF7 cells (65). ATF-2, which binds the CRE site with CREB in MCF7 cells (65), repressed cyclin D1, raising the possibility that ATF-2 may play an intermediary role in repression of cyclin D1 by p16INK4a. Herein, transfection of wild-type CREB enhanced cyclin D1 promoter activity, and a dominant-negative mutant CREB repressed promoter activity 15–20%. A dominant-negative CREB was shown previously to inhibit pp60src-induced cyclin D1 expression in MCF7 cells and point mutation of Ser-133 in CREB was shown to abolish induction by pp60src. The CREB coactivator, CBP, is tethered to the basal transcription apparatus and RNA polymerase II by RNA helicase A (66). The RNA polymerase II carboxyl-terminal domain is activated and phosphorylated by TFIH, which contains cdk7. Furthermore, p16INK4a inhibits carboxyl-terminal kinase activity (61). Together these studies raise the possibility that p16INK4a has the capacity to repress CRE activity of the cyclin D1 promoter through interactions with components of the basal transcription apparatus. Several oncogenic signaling pathways activate cyclin D1 expression through the CRE site, including SV40 small t antigen (67) and activating mutants of pp60src (65). Together, these observations
suggest that the CRE site serves as a common target for activation by oncogenic signals and repression by the tumor suppressor p16INK4a.
p19ARF-repression of cyclin D1 involved a novel cis-element. p53, which has been reported to repress cyclin D1 (68), was induced by p19ARF-transfection, raising the possibility that p53 may play an intermediary role in p19ARF-mediated repression of cyclin D1. ChIP assays herein identified p53 within the context of the local chromatin structure of the cyclin D1 promoter at −1137. Although p53 was shown to repress cyclin D1 promoter activity through more proximal elements (69), these previous experiments were conducted with 66-bp promoter fragments that did not include the more distal elements identified herein. The ChIP assays herein demonstrated p53 at both promoter fragments that did not include the more distal elements (69), these previous experiments were conducted with 66-bp cyclin D1 promoters and distinct DNA sequences.

There is substantial and growing evidence for cross-talk between the p16INK4a and p19ARF. p16INK4a and p19ARF inhibit cyclin D1 promoter activity through select and distinct DNA sequences. p19ARF-mediated repression involves p53 as a common target for activation by oncogenic signals including β-catenin, Src, and the Ras-Raf-MAPK pathway (24–27). The capacity of p16INK4a and p19ARF to repress cyclin D1 may provide a fail-safe mechanism to limit oncogenic responses.

REFERENCES


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