Antiproliferative Autoantigen CDA1 Transcriptionally Up-regulates p21\(^{\text{Waf1/Cip1}}\) by Activating p53 and MEK/ERK1/2 MAPK Pathways*  

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We previously reported that overexpression of cell division autoantigen 1 (CDA1) in HeLa cells arrests cell growth and inhibits DNA synthesis at S-phase (1). Here we show that CDA1-induced arrest of cell growth is accompanied by increases in protein and mRNA levels of the cyclin-dependent kinase (Cdk) inhibitor protein, p21\(^{\text{Waf1/Cip1}}\) (p21). Both p21 induction and cell growth arrest are reversed when CDA1 expression is inhibited. CDA1 also increases p53 protein, but not its mRNA, in a time- and dose-dependent manner. MDM2, a ubiquitin ligase regulating p53 degradation, is inactivated by CDA1, suggesting that p53 protein accumulation is due to decreased protein degradation. Knockdown of p53, using siRNA targeting two sites of p53 mRNA, abrogates transcriptional induction of p21 by CDA1. Deletion of the p53 responsive element in the distal region of p21 promoter attenuates promoter activity in response to CDA1. DNA damage caused by camptothecin treatment increases mRNA and protein levels of CDA1, accompanied by induction of p53. The DNA damage-induced p53 induction is markedly attenuated by CDA1 knockdown. CDA1 induces phosphorylation of ERK1/2 (p44/42), an activity blocked by PD98059 and U0126, inhibitors of the upstream kinase MEK1/2. The MEK inhibitors also block induction of p21 mRNA and abrogate p21 promoter activity stimulated by CDA1. Cell cycle kinases, Cdk1, -2, -4, and -6 are inhibited by CDA1 overexpression. We conclude that CDA1 induces p53- and MEK/ERK1/2 MAPK-dependent expression of p21 by acting through the p53 responsive element in the p21 promoter and that this contributes to its antiproliferative activity.

We previously cloned cell division autoantigen 1 (CDA1)\(^2\) by screening a human testis cDNA expression library using an autoimmune serum from a patient with discoid lupus erythematosus (1). CDA1 has also been described as se20-4 (a cutaneous T-cell lymphoma-associated antigen) (2), DENTT (a TGFB-beta target gene) (3), TSPX (a homologue of TSPY on the X chromosome) (4), and NP79 (a gene differentially expressed in heart samples of common congenital heart defects) (5). A mouse homologue of CDA1 was named CASK-interacting nucleosome assembly protein (CINAP) and was reported as a co-transcription factor in neurons with CASK binding and nucleosome assembly protein activity (6, 7).

CDA1 has 693 amino acid residues with 3 structural domains: an N-terminal proline-rich domain, a central basic domain, and a C-terminal acidic domain. Four nuclear localization signals in the basic and N-terminal proline-rich domains target CDA1 to the nucleolus and nucleolus in transfected mammalian cells (1, 3). Localization of endogenous CDA1/DENTT in either cytoplasm or nucleus or both has also been reported in certain tissues of adult mouse and monkey (8, 9).

CDA1 shares sequence homology with the leukemia protein SET and other proteins including nucleosome assembly proteins (NAPs), TSPY, and TSPY-like proteins (1). These proteins are related to cancer and are directly involved in regulating cell cycle and chromatin remodeling (10–18). SET arrests the cell cycle in G\(_2\)/M-phase by inhibiting the activity of cyclin B/Cdk1 (13). NAP not only acts as an acidic histone chaperone (19) but also mediates chromatin fluidity by incorporating histone variants and assisting nucleosome sliding (20, 21).

We previously reported that CDA1 overexpression, regulated by a Tet-Off expression system, inhibited cell proliferation in a time- and dose-dependent manner (1). DNA synthesis in S-phase cells was also inhibited by CDA1 overexpression. The action of CDA1 in inhibiting cell growth was further supported by the observation that CDA1 was up-regulated in growth-arrested Jurkat T cells (22). The ability of CDA1 to inhibit cell growth required full-length CDA1 and phosphorylation of its two Cdk phosphorylation sites. A CDA1 double mutant with both phosphorylation sites mutated to alanine, failed to inhibit DNA synthesis in S-phase cells (1). In vitro phosphorylation assays demonstrated that cyclin D/Cdk4, cdc2, cdk1, and cdk2 are related to cancer and are directly involved in regulating cell cycle and chromatin remodeling (10–18). SET arrests the cell cycle in G\(_2\)/M-phase by inhibiting the activity of cyclin B/Cdk1 (13). NAP not only acts as an acidic histone chaperone (19) but also mediates chromatin fluidity by incorporating histone variants and assisting nucleosome sliding (20, 21).

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cyclin A/Cdk2, and cyclin B/Cdk1 can phosphorylate CDA1 at either or both phosphorylation sites. Phospho-amino acid analysis confirmed that both sites (serine and threonine) were phosphorylated by cyclin A/Cdk2 in vitro (1).

Cell cycle progression is controlled by kinase activities of Cdkks complexed with specific cyclins at certain cell cycle phases, which are negatively regulated by Cdk inhibitor proteins (CdkIs) belonging to the Cip/Kip and INK4 families. These two families of Cdk inhibitors inactivate different classes of Cdkks (23, 24). The Cip/Kip family member, p21(Waf1/Cip1) (p21) was first identified in a quaternary complex containing D cyclin, Cdk, and PCNA (25), and shown to inhibit cell proliferation (26) and activities of several cyclin-Cdk complexes in vitro (26, 27). Transcriptional regulation of the p21 gene is controlled by the tumor suppressor protein p53 acting on the p53 responsive element in the distal region of the p21 promoter in response to intracellular signals such as DNA damage (26, 28–32). TGF-β transcriptionally regulates p21 by the TGF-β responsive element at the proximal region, which does not necessarily require functional p53 in HaCat cells (33, 34). The intracellular signaling pathway MEK/ERK has been reported to be required for up-regulation of p21 in response to stimulation by several factors including TGF-β (35–41).

In this study, we show that CDA1 induces p21 transcriptional activation and expression, an observation consistent with our previous report that CDA1 overexpression inhibits DNA synthesis and cell proliferation (1). Further, we show that CDA1-mediated expression of p21 is regulated by p53 and the MEK/ERK1/2(p44/p42) signaling pathway and that these actions are most likely mediated through the p21 promoter.

**EXPERIMENTAL PROCEDURES**

Antibodies, Plasmids, and Reagents—The rabbit antibody raised to human CDA1 has been described previously (1). Antibodies to CDK1 (SC-8395), CDK2 (SC-6248), CDK4 (SC-260), CDK6 (SC-7961), p21 (SC-397), p53 (SC-126), and ERK1/2 (p42/p44) (SC-93) were from Santa Cruz Biotechnology. Antibodies to phospho-ERK1/2 (p42/p44) (Thr202/Tyr204) (9106), phospho-MDM2 (Ser166) (3521), phospho-Akt (Ser473) (4058), and Akt (9272) were from Cell Signaling Technology. The MEK inhibitor, PD98059 was from Sigma and U0126 from Cell Signaling Technology. Camptothecin (CPT) was purchased from Sigma Aldrich Pty Ltd. pGEX-Rb-C150 was constructed to express GST-Rb protein to be used as a substrate of Cdk4 and Cdk6 for the in vitro phosphorylation assay. cDNA encoding the C-terminal 152 amino acids of retinoblastoma protein was amplified by PCR from HeLa cell cDNA and cloned into a pGEX 4T-3 plasmid (Promega) at the BglII site. The primers used for PCR were: forward, 5′-TCCACTagtATCTATGCTGCTTGCAGGAAA-3′ and reverse, 5′-CACAAaGATCGACTTCGCAGCTGCACAC-3′. The distal region (90 bp) of the promoter containing the p53 responsive element (29) was removed by digestion with SacI and religated to generate p21(p53-)Luc construct.

Cell Culture—The HeLa cell line stably expressing CDA1 with Tet-Off control has been described previously (1). Approximately 2 × 10⁵ cells per 10-cm dish were plated in DMEM medium plus 10% FBS and 5 ng/ml doxycycline and incubated at 37 °C with 5% CO₂ overnight. Attached cells were washed twice with phosphate-buffered saline and replaced with DMEM medium plus 10% FBS with or without the desired concentrations of doxycycline for a specified period of time to over-express the CDA1 transgene.

Gene Knockdown by Retroviral-transduced siRNA—The retroviral siRNA constructs were made by cloning complementary oligonucleotides into pSUPER-Retro-puromycin (OligoEngine, Seattle, WA) and pSUPER-Retro-EGFP plasmids (provided by Dr. Gianpietro Dotti, Baylor College of Medicine) (42), and transfected into RetroPack PT67 cell line (Clontech Laboratories, Inc) to produce viruses in the medium. The retroviruses were used to transduce HeLa cells or the HeLa cell line expressing CDA1 under Tet-Off control, which were then selected with puromycin (5–10 μg/ml) for at least 2 weeks. The siRNA target sequences are: CDA1 siRNA, 5′-GACATATCTCCATGGGCTA-3′; p53 siRNA 825, 5′-GACATCTTATCAGGATGGA-3′ and p53 siRNA 1026, 5′-GACCTCGTGTGAATCTAC-3′.

Kinase Assay—Cyclin-dependent kinase complexes were immunoprecipitated from the cell lysate using antibodies to the particular Cdk. Cells were lysed with lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science)) and 500 μg of total supernatant proteins were incubated with 200 ng of antibodies in a final 1 ml of lysis buffer by rotating at 4 °C overnight. The following morning, 50 μl of 50% protein G slurry (Amersham Biosciences Cat. 17-0618-01) was added and rotated at 4 °C for 1.5 h. Immune complexes were pelleted by centrifugation, washed five times with lysis buffer and twice with phosphorylation buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 50 μM ATP) and resuspended in 100 μl of phosphorylation buffer. To determine the kinase activity of immunoprecipitated Cdkks, 5 μl of the IP beads were mixed with 1 μg of substrate protein, histone H1 for Cdk1 and Cdk2 or GST-Rb for Cdk4 and Cdk6, 2 μl [γ-³²P]ATP (PerkinElmer Life Sciences) in the final 30 μl of phosphorylation buffer, and incubated at 30 °C for 30 min. The reaction was stopped by addition of 30 μl of 2× reducing SDS-PAGE sample buffer and boiling for 5 min. After chilling on ice briefly, the beads were pelleted and 10 μl of supernatant loaded for SDS-PAGE. The gel was fixed and dried before exposure to x-ray film.

Gene-specific mRNA Quantitation by Real-time RT-PCR—Gene-specific mRNA was quantitatively determined by reverse transcription of 2 μg of total RNA using random primers followed by real-time PCR using gene-specific primers and probes, their design based on cDNA sequences of the genes of interest. PCR products were quantitatively determined at each PCR cycle using 7500 Fast Real-Time PCR System (Applied
p21 Induction by CDA1

**FIGURE 1. CDA1 overexpression increased protein and mRNA levels of p21.** CDA1 expression was induced in the HeLa cell line expressing CDA1 under Tet-Off control, by withdrawal of doxycycline from the culture medium for up to 72 h (top panels), or by culturing cells in decreasing doxycycline concentrations for 72 h (bottom panels). A, approximately 20 μg of total cellular lysate protein per lane was used for Western blot analysis to show protein levels of CDA1 and p21. The blots were re-probed for α-tubulin to indicate equal loading per lane. B, CDA1 (left panels) and p21 (right panels) mRNA levels were determined by quantitative real-time RT-PCR and shown as fold change (arbitrary unit). Significant difference relative to the cells with no CDA1 induction, 0 h (top panels) or 5 ng/ml doxycycline (bottom panels) is indicated by an asterisk representing p < 0.05 or double asterisks representing p < 0.01. S.E. (n = 6) is shown as an error bar.

Biosystems, Foster City, CA) and normalized against endogenous 18 S ribosomal RNA using TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems, Foster City, CA). Results were analyzed and shown as fold change (arbitrary unit) relative to a control group. Gene-specific primers and probes were: CDA1, probe: 6-carboxyfluorescein (FAM)-5′-TTCTTGCTGCTCAAGC-3′-6-carboxy-tetramethylrhodamine (TAMRA) (quencher), forward primer: 5′-AACATCAAGGAGCAGTAGAAGC-3′, reverse primer: 5′-GAAAGGCTCTCAGCTGACATGGAT-3′; p21 probe: 6-FAM-5′-CAGACAGCATGACAG-3′-TAMRA, forward primer: 5′-CTGAGACTCTAGGGTCGA-3′, reverse primer: 5′-CCGGCTTTGAGTTGGTAAGA-3′; p53 probe: 6-FAM-5′-CCGCTTGCGCTCCTG-3′-TAMRA, forward primer: 5′-AGAAAACTACAGGGGCA-GCTA-3′, reverse primer: 5′-TGGCTGTCCAGAATGCAGG-3′.

**Thymidine Incorporation and Detection**—1.2 × 10⁶ cells per well were seeded in 6-well plates and incubated under specified conditions for a desired period of time. Cells were washed with DMEM and refilled with 2 ml of DMEM medium containing 2 μl of [³H]thymidine (PerkinElmer Life Sciences) per well and incubated for 2 h at 37 °C. Incorporated [³H]thymidine was counted for 2 min after mixing 0.5 ml of lysed cell sample, 10 ml of Insta-Gel Plus (PerkinElmer Life Sciences), and 0.5 ml of 1 M HCl.

**Luciferase Reporter Assay**—CDA1 Tet-Off HeLa cells (2 × 10⁵ cells) were co-transfected with p21-Luc or p21(p53)-Luc or pGL3-BASIC (15 μg) and CMV-β-galactosidase (3 μg) plasmids by electroporation (0.26 kV and 960 μF), plated in 6-well plates and incubated in the presence of doxycycline (5 ng/ml) overnight. Cells were washed twice with phosphate-buffered saline and refilled with DMEM plus 10% FBS with or without 5 ng/ml doxycycline and incubated for 48 h. Luciferase and β-galactosidase activities of each sample were analyzed using the Luciferase Assay system (Cat. E1501) and the β-galactosidase Enzyme Assay (Cat. E2000) from Promega following the manufacturers’ instructions. Luciferase activity was normalized against β-galactosidase activity for each sample and compared between the cells with CDA1 transgene turned on and off.

**RESULTS**

**CDA1 Overexpression Induces p21**—Previously we showed that CDA1 inhibited DNA synthesis at S-phase (1). Therefore, we examined the effect of CDA1 overexpression in HeLa cells on protein levels of p21, the Cip1/Kip1 Cdk inhibitor that negatively regulates S-phase entry and progression. CDA1 levels increased over 72 h following doxycycline withdrawal in the Tet-Off system (Fig. 1A). p21 was not detectable at 0 and 6 h, but became detectable at 12 h and peaked at 48 h (Fig. 1A, top panel). p21 induction by CDA1 was further explored in a study where CDA1 transgene expression was increased by decreasing doxycycline concentrations for 72 h (Fig. 1A, bottom panel). CDA1 was expressed at its highest level in the absence of doxycycline and at its lowest level with 5 ng/ml doxycycline. When doxycycline concentration was decreased below 5 ng/ml, CDA1 protein levels progressively increased. In these samples, p21 was induced when CDA1 was expressed at a higher level with 0.5 ng/ml doxycycline and reached its highest level with 0.13 ng/ml doxycycline and concentrations further decreased (Fig. 1A). These data demonstrate that CDA1 regulated induction of p21 in a concentration-dependent manner.

**p21 Expression Is Caused by Increased Gene Transcription**—We then examined whether the increased accumulation of p21 induced by CDA1 is the result of increased gene transcription. CDA1 mRNA, assessed by real-time RT-PCR, increased 40-, 60-, and 120-fold at 6, 12, and 24 h, respectively, after doxycycline withdrawal (Fig. 1B). High levels of CDA1 mRNA were maintained for up to 72 h. Following the increase in CDA1 mRNA levels, p21 mRNA levels started to increase at 12 h after doxycycline withdrawal, being over 3- to 10-fold increased compared with controls at 12, 24, and 48 h, respectively (Fig. 1B). The mRNA levels of both CDA1 and p21 in the cells in which CDA1 transgene was turned off were unchanged.
at all the time points studied (data not shown), suggesting that induction of p21 was CDA1-specific.

The dependence of transcriptional induction of p21 on CDA1 protein levels was further evaluated by increasing levels of CDA1 for 72 h using doxycycline in decreasing concentrations. When CDA1 mRNA was induced 43-, 68-, and 105-fold, respectively, p21 mRNA was concomitantly increased 1.7-, 2.7-, and 7.3-fold (Fig. 1A). Thus, CDA1 induces p21 mRNA in a concentration-dependent manner. These studies showed that p21 mRNA levels were dependent on the protein and mRNA levels of CDA1 (Fig. 1A and B).

**p21 Induction and Cell Growth Arrest Are Reversed by Turning Off CDA1 Transgene**—CDA1 protein expression in the Tet-Off HeLa cells incrementally increased up to 48 h after doxycycline withdrawal (Figs. 1A and 2A). Re-addition of doxycycline after 48 h to inhibit CDA1 transgene expression resulted in a gradual decrease in CDA1 protein levels over 4 days (Fig. 2A). p21 mRNA levels in these cells closely correlated with CDA1 protein levels, with an initial increase to peak levels at 48 h and then a gradual decrease after CDA1 transgene expression had been inhibited (Fig. 2B). Cells with CDA1 transgene turned off proliferated rapidly, as assessed by [3H]thymidine incorporation, during the initial 72 h and then at a constant rate over the following 2 days (Fig. 2C, open square). When CDA1 transgene was turned on for the initial 48 h, cell proliferation was inhibited with an unchanged thymidine incorporation rate (Fig. 2C, filled circle). However, turning off CDA1 transgene expression after 48 h (Fig. 2C, open circle) reversed the cell growth arrest, and cells began proliferating again 3 days later, at the time point of 120 h, followed by even more rapid proliferation over the following 2 days (Fig. 2C). At 120 and 144 h, CDA1 protein levels had decreased to a lower level (Fig. 2A). This demonstrated that the cells with CDA1 overexpression for at least 48 h were still viable and that the growth arrest was reversible. Furthermore, this experiment confirmed that p21 induction and cell growth arrest were regulated by CDA1 in a dose-dependent and reversible manner.

**CDA1 Regulates p53 Protein Accumulation by Inactivating MDM2**—As transcriptional regulation of the p21 gene is controlled by the tumor suppressor protein p53 (26, 28–32), we examined the effects of CDA1 overexpression on protein and mRNA levels of p53. CDA1 protein levels were markedly increased 6 h after CDA1 transgene was turned on and further increased with time, peaking at 48 and 72 h (Fig. 3A, top panel), consistent with the result shown in Fig. 1A. Induction of p53 protein levels showed a close correlation to CDA1 levels (Fig. 3A, top panel). In contrast, levels of phosphorylated MDM2 at Ser166 (43, 44) inversely correlated with the CDA1 levels (Fig. 3A, top panel), indicating a CDA1-dependent inactivation of MDM2. Levels of phosphorylated Akt (Ser473) (pAkt), and Akt. Blots were re-probed for α-tubulin to indicate equal loading per lane. B, quantitative real-time RT-PCR showing relative mRNA levels of p53 in the time course samples.

**FIGURE 2. Induction of p21 and cell growth arrest by CDA1 is reversible.** CDA1 transgene was induced by withdrawing doxycycline (dox− (A); ○ (B and C)) from the medium for 48 h and then inhibited by adding 5 ng/ml doxycycline (dox+ (A); □ (B and C)). Cells were harvested at the specified time points to measure CDA1 expression levels by Western blot analysis (A), p21 mRNA levels by real-time RT-PCR (B), and cell proliferation by [3H]thymidine incorporation (C). For the cell proliferation assay, a control group of cells with the CDA1 transgene turned off during the entire time course (□ in all panels) is also shown (C).

**FIGURE 3. CDA1 increases p53 protein levels by inactivating MDM2.** CDA1 transgene expression was regulated by doxycycline withdrawal for specified times up to 72 h (A, top panel and B) or by decreasing doxycycline concentrations over 72 h (A, bottom panel) as described in the legend of Fig. 1A. Western blot analysis shows protein levels of CDA1, p53, phosphorylated MDM2 (Ser166) (pMDM2), phosphorylated Akt (Ser473) (pAkt), and Akt. Blots were re-probed for α-tubulin to indicate equal loading per lane. B, quantitative real-time RT-PCR showing relative mRNA levels of p53 in the time course samples.
accompanied by a progressive increase in p53 protein levels (Fig. 3A, bottom). mRNA levels of p53 were not changed in this time course study (Fig. 3B), suggesting that the effect of CDA1 on p53 was only at the post-translational level.

p53 Is Required for Transcriptional Induction of p21 by CDA1—To confirm the requirement of p53 for p21 induction by CDA1 we generated four retrovirus transduced cell pools expressing hairpin-structured siRNA targeting CDA1 or p53. We then examined the level of protein expression of these 2 proteins in the cell pools (Fig. 4A). The CDA1 transgene was turned on by doxycycline withdrawal or turned off with 5 ng/ml doxycycline added to the medium for 48 h. CDA1 overexpression was seen in the cells transduced with vector control virus and two different p53 siRNA viruses. The CDA1 siRNA retrovirus-transduced cells showed a marked reduction in endogenous CDA1 level with the CDA1 transgene turned off. In cells with the CDA1 transgene turned on, CDA1 expression level was also markedly decreased compared with vector control and p53 siRNA retrovirus-transduced cells. p53 levels in cells with CDA1 knockdown were much lower than that in the vector control cells. CDA1-dependent p53 induction was also inhibited by CDA1 knockdown when the CDA1 transgene was turned on. These results indicate a role for CDA1 in regulating p53 protein levels.

Western blot analysis also showed that both siRNA species of p53 (siRNA 825 and siRNA 1026) targeting two sites in the coding region reduced p53 to undetectable levels, even in the cells with the CDA1 transgene turned on. By contrast, in vector control cells, induction of p53 expression was seen when the CDA1 transgene was turned on (Fig. 4A), consistent with the results shown in Fig. 3A.

siRNA knockdown of p53 markedly reduced p21 mRNA levels, compared with the no virus and vector virus transduced control cells, as determined by quantitative real-time RT-PCR (Fig. 4B). p21 mRNA induction by CDA1 overexpression seen in control cells was also attenuated or totally blocked by p53 siRNA knockdown (Fig. 4B), demonstrating the requirement of p53 for p21 induction by CDA1. The difference in the magnitude of p21 mRNA reduction in p53 siRNA 825 and 1026 transduced cells can be attributed to variation in p53 knockdown by these two different siRNA retroviruses. Indeed, quantitative real-time PCR measurements of p53 gene expression showed that siRNA 825 and 1026 reduced p53 mRNA to 57 and 9%, respectively, compared with control cells (Fig. 4C). The p53 mRNA level was not changed in the cells when CDA1 was overexpressed (Fig. 4C), consistent with results shown in Fig. 3B.

p53 Responsive Element Is Required for Activation of p21 Promoter by CDA1—We further investigated transcriptional induction of the p21 gene by examining the effect of CDA1 on p21 gene promoter activity. The human p21 gene promoter was activated by CDA1 overexpression in HeLa cells as shown by luciferase activity of a human p21 gene promoter (2.4 kb)-driven luciferase reporter construct. Basal promoter activity of p21-Luc was ~20-fold (1 versus 0.05), compared with the pGL3-BASIC control, a promoterless luciferase gene construct (Fig. 5A). When CDA1 transgene was turned on by doxycycline withdrawal for 48 h, p21-Luc activity was further increased by 4.88-fold (Fig. 5A), suggesting that CDA1 induces p21 gene and protein levels by increasing p21 gene promoter activity. The induced promoter activity by CDA1 was blocked by the MEK inhibitors PD98059 and U0126 (Fig. 5B), consistent with the blocked induction of p21 mRNA by CDA1 shown in Fig. 7. This

FIGURE 4. CDA1 regulates protein levels of p53 required for the action of CDA1 in inducing p21 gene expression. Retrovirus-transduced stable cell pools expressing CDA1 siRNA, p53 siRNA 825, p53 siRNA 1026, and a vector virus control cell pool (Vec) were derived from the HeLa cell line expressing CDA1 under Tet-Off control. They were cultured for 48 h with CDA1 transgene turned on by doxycycline withdrawal (+) or turned off with 5 ng/ml doxycycline (−). A, Western blot analysis shows protein levels of CDA1 and p53. Blots were re-probed for α-tubulin to indicate equal loading per lane. B, quantitative real-time RT-PCR shows relative mRNA levels of p21 and p53.
CDA1 is an Upstream Regulator of p53 in CPT-induced DNA Damage—We used camptothecin (CPT) to treat HeLa cells in order to examine the physiological role of CDA1 in regulating p53 induction in response to CPT-induced DNA damage. Western blot analysis showed that CDA1 and p53 protein levels were markedly increased after CPT (1 μM) treatment for 12 and 24 h (Fig. 6A) in the vector retrovirus-transduced HeLa cells. In CDA1 siRNA retrovirus-transduced cells treated with CPT, CDA1 protein level did not increase, and p53 induction was markedly attenuated (Fig. 6A). CDA1 mRNA levels increased ~3-, 5-, and 7-fold after CPT treatment for 8, 12, and 24 h, respectively (Fig. 6B) in vector retrovirus-transduced HeLa cells, indicating that CPT treatment up-regulated CDA1 protein levels by activating gene expression. In the CDA1 siRNA retrovirus-transduced cells, CDA1 mRNA levels were lower than vector control cells before and after CPT treatment (Fig. 6B). A slight induction of CDA1 mRNA levels after CPT treatment for 8, 12, and 24 h, compared with that before CPT treatment and the vehicle control, was observed. This was probably due to incomplete knockdown of CDA1 mRNA by this siRNA and may explain the slight induction of p53 protein levels at 12 and 24 h in these cells shown in Fig. 6A. These results clearly show that CDA1 is essential for p53 induction in response to CPT-induced DNA damage, and the magnitude of p53 induction is dependent on the levels of CDA1 that were induced.

Activation of the MEK/ERK MAPK Pathway Is Required for Induction of p21 mRNA—We investigated the involvement of the MEK/ERK MAPK pathway in CDA1-induced p21 gene expression, because previous studies have implicated this signaling pathway in p21 expression (35–41,46). Our results demonstrated that CDA1 overexpression activated the ERK1/2 (p44/42) MAPK pathway, as determined by an antibody recognizing phosphorylated ERK1/2, phosphorylated at Thr202 and Tyr204. ERK1/2 MAPK was activated 12 h after CDA1 transgene induction (Fig. 7A), at a time when p21 gene expression was concomitantly induced (Figs. 1B and 2B). Activation of ERK MAP kinase was maintained at a high level for an additional 12 h and then gradually decreased, but remained elevated, albeit at a lower level at 48 and 72 h (Fig. 7A). The MEK inhibitors, PD98059, at concentrations of 10, 25, and 50 μM (Fig. 7B, top panel) and U0126 at 1, 5, and 10 μM (Fig. 3B, bottom panel) inhibited ERK1/2 MAP kinase phosphorylation when added to cells at the same time as doxycycline was withdrawn to induce CDA1 overexpression. Both MEK inhibitors blocked the action of CDA1 in inducing p21 (Fig. 7C), suggesting that activation of the MEK/ERK MAPK pathway is also required for CDA1 to induce mRNA levels of p21. This is consistent with the require-
ment of this signaling pathway in the activation of p21 gene promoter by CDA1, as shown in Fig. 5B.

**Kinase Activity of Cdk 1, 2, 4, and 6 Is Inhibited in Cells with CDA1 Overexpression**—The finding that CDA1 overexpression resulted in cell growth arrest (1) and induction of the CDK inhibitor protein, p21, raised the possibility that the activity of CDKs might be inhibited in these cells. Cyclin-dependent kinases 1, 2, 4, and 6 were immunoprecipitated from cells following CDA1 induction for 72 h as well as an appropriate control, from cells where CDA1 was not induced. Immunoprecipitated Cdk proteins were examined by Western blot to confirm similar levels of Cdk proteins in the immunoprecipitates from cells with the CDA1 transgene turned on or off (Fig. 8). Kinase activities were then measured by assaying their activities against their respective substrates (histone H1 for Cdk 1 and 2, or retinoblastoma protein (p-Rb) for Cdk 4 and 6) (Fig. 8). The activities of all the examined Cdk1, 2, 4, and 6 were inhibited, albeit a residual activity of Cdk1 was still visible in cells where CDA1 had been induced when compared with cells where CDA1 had not been induced (Fig. 8). No detectable CDK protein or Cdk activities were present in control immunoprecipitates using control antibodies (data not shown).

**DISCUSSION**

We have previously generated a Tet-Off HeLa cell line where CDA1 levels can be regulated by doxycycline concentrations (1). Using this cell line, we provide in this series of experiments compelling data that implicates CDA1 in the regulation of the transcription and expression of p21, a Cdk inhibitor protein of the Cip/Kip family. First, a progressive time- and dose-dependent increase in levels of CDA1 protein and mRNA is accompanied by a corresponding progressive increase in gene and protein expression of p21 (Fig. 1). Second, the increase in p21 protein and mRNA levels is delayed relative to increase in CDA1 suggesting that this phenomenon is secondary to increased CDA1 levels. Third, a progressive increase followed by a subsequent decrease in
CDA1 levels, was associated with a similar rise and fall in p21 levels (Fig. 2). Finally, CDA1 overexpression activated the p21 promoter in a luciferase reporter assay (Fig. 5).

Inhibiting CDA1 transgene overexpression after 48 h resulted in a gradual decrease in CDA1 protein levels, indicating a relatively long half-life of CDA1 protein in these cells. When CDA1 protein and p21 mRNA levels dropped to a level similar to that seen endogenously, cell growth arrest was released, allowing cells to proliferate again. This indicates that the CDA1-overexpressing cells remained viable, consistent with our previous report (1). More importantly, this finding demonstrated that cell growth arrest and p21 induction were both quantitatively dependent on CDA1 expression.

Gene transcription of p21 is known to be induced by intra-cellular signaling, such as DNA damage, in a p53-dependent pathway (28–30). We observed that CDA1 overexpression induces expression of p53, an effect that was lost with siRNA knockdown of CDA1. The observation that CDA1 inactivates MDM2, the ubiquitin ligase for p53 degradation (45), suggests that the increase in p53 levels is due to increased protein stability. These data are consistent with a lack of an increase in mRNA levels of p53 with CDA1 overexpression. The ability of MDM2 to degrade p53 is positively regulated by phosphorylation at Ser166 by Akt (43, 44). Our finding of CDA1-dependent inactivation of Akt suggests a mechanism contributing to MDM2 inactivation thereby increasing p53 stability. Structural studies of the p21 gene promoter have revealed a major p53 responsive element located −2.4-kb upstream of the p21 gene transcription site (30). This has been further confirmed recently by global screening of p53 binding sites in the human genome (47).

However, the target of activated ERK1/2 MAPK on the p21 gene transcription site (30). This has been further confirmed recently by global screening of p53 binding sites in the human genome (47).

The finding of CDA1-dependent induction of p53 indicated a possibility that CDA1 might play a key role as an upstream regulator of p53 in DNA damage response. Several reports have implicated CDA1 as an important mediator in cell cycle arrest, or apoptosis (48–54). CPT is an inhibitor of DNA topoisomerase I, which traps the enzyme in the cleavable complexes within the transcribed region of the chromosome, leading to irreversible DNA breaks (55–57). After CPT treatment, CDA1 mRNA and protein expression levels were indeed increased with concomitant induction of p53 protein (Fig. 6). Further, knockdown of CDA1 attenuated p53 induction by CPT-induced DNA damage (Fig. 6). This is the first report demonstrating the function of CDA1 as an essential upstream regulator of p53 in DNA damage response, though it is still unclear as to the exact mechanism whereby CDA1 is up-regulated by DNA damage.

ATM-dependent ERK1/2 MAPK activation has previously been reported, in parallel with p53 stabilization, in the DNA damage response, and contributes to p21 induction and G1 arrest caused by DNA damage (58). Our findings that CDA1 activates both p53 and ERK1/2 MAPK pathways suggest that CDA1 is a key molecule in the DNA damage response, acting as a regulator of both p53 and ERK1/2 MAPK pathways.

Mild stimulation of the residual activity of p21(p53−)-Luc by CDA1 overexpression in cells could be via other responsive elements present in the downstream region. Our data suggest that the MEK/ERK1/2 MAPK pathway may contribute to this residual activity. The suggestion is supported by the observation that inhibition of ERK1/2 MAPK activation by the two MEK inhibitors, PD98059 and U0126, abrogated not only transcriptional induction of p21 but also blocked p21 promoter activity stimulated by CDA1. These data are consistent with previous reports of induction of p21 gene expression by this signaling pathway (35–38, 40, 46). Thus, it is likely that both p53 and activated ERK1/2 MAPK are involved in binding to the distal region of p21 promoter, thereby activating the p21 gene. However the target of activated ERK1/2 MAPK on the p21 gene
p21 Induction by CDA1

promoter remains unknown. Phosphorylation of ERK1/2 induced by CDA1 overexpression implicates CDA1 in activating the ERK1/2 MAPK signaling pathway. The observation of a gradual decrease in phosphorylation of ERK1/2 while CDA1 is still expressed at a high level probably reflects the negative regulation of ERK1/2 by dual-specificity MAP kinase phosphatases (60–62). Nevertheless, this result demonstrates that CDA1 is able to activate ERK1/2 and to maintain ERK1/2 phosphorylation at a level significantly higher than the basal level. Effective inhibition of CDA1-dependent ERK1/2 activation by two inhibitors of the upstream kinase MEK, PD98059 and U0126, suggest that CDA1 overexpression modulates upstream kinases such as MEK, Raf, Ras, and possibly cell surface receptor kinases linked to this pathway. Considering the intracellular nuclear localization of CDA1, it seems likely that the action is indirect via other proteins influenced by CDA1. However, it is possible that CDA1 may be able to shuttle between the cytoplasm and nucleus since endogenous CDA1, also known as DENTT, has been detected in the cytoplasm, nucleus or both in certain tissues of adult mouse and monkey (8, 9).

CDA1-induced expression of p21 is accompanied by inhibition of the activity of G1/S-, S-, and G2/M-phase Cdk, including cyclin E/Cdk2, cyclin A/Cdk2, and cyclin B/Cdk1. The data are consistent with our previous report that CDA1 overexpression inhibits DNA synthesis (1), an activity dependent on S-phase Cdk. However, CDA1 overexpression also inhibits the activities of G1-phase Cdk, namely Cdk4 and Cdk6. These activities cannot be attributed solely to induction of p21 expression as p21 plays a positive role in regulating activities of the D-type cyclins-Cdk 4/6 (59, 68–70), by indirect via other proteins influenced by CDA1. Considering the intracellular localization of CDA1, it seems likely that the action is indirect via other proteins influenced by CDA1. However, it is possible that CDA1 may be able to shuttle between the cytoplasm and nucleus since endogenous CDA1, also known as DENTT, has been detected in the cytoplasm, nucleus or both in certain tissues of adult mouse and monkey (8, 9).

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