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Research Article

Cyclin A1 is a p53-induced gene that mediates apoptosis, G2/M arrest, and mitotic catastrophe in renal, ovarian, and lung carcinoma cells

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Abstract. We were the first to identify cyclin A1 as a p53-induced gene by cDNA expression profiling of p53-sensitive and -resistant tumor cells [Maxwell S. A. and Davis G. E. (2000) Proc. Natl. Acad. Sci. USA 97, 13009–13014]. We show here that cyclin A1 can induce G2 cell cycle arrest, polyploidy, apoptosis, and mitotic catastrophe in H1299 non-small cell lung, TOV-21G ovarian, or 786-0 renal carcinoma cells. More cdk1 protein and kinase activities were observed in cyclin A1-induced cells than in GFP control-induced cells. Thus, cyclin A1 might mediate apoptosis and mitotic catastrophe through an unscheduled or inappropriate activation of cdk1. Two primary renal cell carcinomas expressing mutated p53 exhibited reduced or absent expression of cyclin A1 relative to the corresponding normal tissue. Moreover, renal carcinoma-derived mutant p53s were deficient in inducing cyclin A1 expression in p53-null cells. Cyclin A1 but not cyclin A2 was upregulated in etoposide-treated tumor cells undergoing p53-dependent apoptosis and mitotic catastrophe. Forced upregulation of cyclin A2 did not induce apoptosis. The data implicate cyclin A1 as a downstream player in p53-dependent apoptosis and G2 arrest.

Keywords. Apoptosis, mitotic catastrophe, p53, cyclin A1, proline oxidase, cdk1.

Introduction

The product of the p53 gene plays pivotal roles in several biological processes that are important in reducing the tumorigenic potential of cells. The protein mediates cell growth arrest by controlling cell-cycle checkpoints, induces apoptosis, and plays a key role in senescence and differentiation [1]. Many studies have implicated p53 in genomic stability, surveillance of DNA damage, and DNA repair, which are related to its control over cell growth. The p53 protein functions as a sequence-specific DNA-binding factor and can transactivate genes whose promoters contain a p53 response element [1]. Many genes have been identified as p53-induced genes (PIGs) that play roles in cell-cycle control, differentiation, DNA repair, angiogenesis, and apoptosis. We have identified some novel PIGs using the DECV cell line, which was selected for resistance to p53-mediated apoptosis by repeated rounds of infection with a recombinant p53 adenovirus [2]. Gene expression profiling by cDNA microarray identified a number of genes that were differentially expressed between p53-sensitive ECV-304 and p53-resistant DECV cells [2]. From this analysis, we were the first to identify cyclin A1 as a PIG [2]. We identified a number of other PIGs including the proline oxidase gene, which encodes a mitochondrion-localized enzyme that induces apoptosis [2].

The induction of the cyclin A1 gene by p53 most likely is indirect, mediated through a protein-protein interaction, since the cyclin A1 gene promoter contains no p53

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consensus binding sites [3]. Moreover, the transcriptional upregulation of the cyclin A1 gene by p53 was dependent on Sp1 and the presence of an Sp1 GC box surrounded by sequences that are highly conserved between the murine and the human cyclin A1 promoter [3]. Cyclin A1 was the only cyclin to be induced by γ-irradiation and p53, whereas cyclin A2 expression was consistently repressed. Thus, in circumstances that require double-strand (ds) break repair, cyclin A1 is induced, whereas cyclin A2 is downregulated. Moreover, cyclin A1-deficient MEF cells, which expressed cyclin A2, were deficient in ds DNA break repair [3].

A number of studies into the function of cyclin A1 suggested that this gene could be important for p53-mediated regulation of the cell cycle and induction of apoptosis. Cyclin A1 plays a role in cell-cycle regulation in both somatic and germ-line cells. Meiotic cells (oocytes and spermatocytes) express cyclin A1 [4], and high-level expression in the healthy organism is restricted to pachytene spermatocytes undergoing meiosis [5]. Cyclin A1 may function as the M-phase cyclin because the progression of spermatogenesis is arrested before the second meiosis in cyclin A1-deficient mice [6]. In further support of this model, male mice homozygous for a mutated allele of the cyclin A1 gene are sterile due to a block in cell-cycle progression before the first meiotic division [7]. Meiosis arrest in these cyclin A1 mutant mice is associated with desynapsis abnormalities, lowered maturation promoting factor activity, and apoptosis [7].

Cyclin A1 has been reported to be preferentially expressed during the meiotic cell cycle, whereas cyclin A2 is preferentially expressed during the mitotic cell cycle [8]. Thus, cyclin A1 has been proposed to regulate M phase in the meiotic cell cycle, whereas cyclin A2 regulates S and M phases in the mitotic cell cycle. These differences in the expression patterns of cyclin A1 and A2 may, therefore, be involved in the meiotic-mitotic transformation of cell cycle regulation after fertilization. However, another study has implicated a role for cyclin A1 in the mitotic cell cycle as well as in addition to its functions in meiosis [9]. Cyclin A1-dependent kinase complexes contain both cdk1 and cdk2 components and cyclin A1 plays an essential role in initiating the activation of cyclin B1/cdk1 kinase at the meiotic G2-M transition of male germ cells [10]. However, both cyclins A1 and A2 appear to prefer cdk2 as a partner in testicular cell lysates [5, 6, 11].

Abnormal expression of cyclin A1 has been associated with tumorigenesis. Human cyclin A1 is highly expressed in several types of acute myelogenous leukemia (AML) [12, 13]. Abnormal myelopoiesis and AML developed in transgenic mice that overexpressed cyclin A1 [14]. Various AMLs have been associated with high levels of C-*MYB* [15], *PML-RAR* or *PLZF-RAR* [16], and these proteins are known transcriptionally to enhance the expression of cyclin A1. Elevated levels of cyclin A1 in non-seminomatous male germ cell tumors, and its absence in carcinoma *in situ* and seminomas suggest a role for the cyclin in tumor transformation and progression, and may be relevant to clinical prognosis [17]. Another study reported that cyclin A1 is highly expressed in aggressive testicular germ cell tumors [18]. Cyclin A1 contributed to G1 to S cell cycle progression in somatic cells by enhancing S phase entry, which is consistent with an oncogenic function [19].

In contrast to the above observations implicating cyclin A1 in oncogenic activities in leukemia and testicular cancers, other studies indicate a potential tumor suppressor role for the protein in nasopharyngeal carcinomas, colon carcinomas, and head and neck squamous cell cancers (HNSCC). First, the expression of cyclin A1 has been found to be downregulated in nasopharyngeal carcinoma and HNSCC [20, 21]. Second, the cyclin A1 promoter, similar to a number of other critical tumor suppressor genes, is frequently hypermethylated in colon cancer and HNSCC [22]. Third, an inverse relationship between cyclin A1 promoter methylation and p53 mutation status has been observed in HNSCC [20]. Fourth, evidence has been provided for a novel pathway linking p53 activity with cyclin A1 induction and subsequent ds DNA break repair [3]. Cyclin A1 was found to interact with the Ku70 and Ku80 proteins [3], which function in DNA replication, recombination in B and T cells, and DNA repair [23].

We report here the characterization of cyclin A1 as a p53-induced target gene with respect to apoptosis and mitotic catastrophe. We provide evidence that cyclin A1 can mediate a G2-arrest and promote apoptosis in renal, ovarian, and lung carcinoma cells. Cyclin A1 might play a role in renal tumorigenesis, since its expression was reduced or absent in two primary renal cell carcinomas that expressed mutated p53s (T4p53 and T7p53). The DNA-damaging agent, etoposide, upregulated both p53 and cyclin A1. A cyclin A1 small interfering interfering RNA (siRNA) repressed etoposide- and p53-induced upregulation of cyclin A1 and induction of apoptosis and mitotic catastrophe. Based on our observations, we propose that cyclin A1 plays a role in p53-mediated apoptosis, G2 arrest, and mitotic catastrophe.

Materials and methods

Antibodies, plasmids, and recombinant adenoviruses. The p53 monoclonal antibody, Bp53-12, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the M30 antibody specific for the caspase-cleaved form of cytokeratin 18 from Roche (Indianapolis, IN), the actin monoclonal antibody (N350) from Amersham Biosciences (Piscataway, NJ), a rabbit anti-α-tubulin antibody (B-5-1-2) from Sigma-Aldrich Biotechnology (Milwaukee, WI), a monoclonal antibody to cyclin A1 (B88-2) from BD Pharmingen (San Diego, CA), a monoclonal antibody to

cyclin A2 (E67.1) from eBioscience (San Diego, CA), and antibodies to cyclin B1 and cdk1 were obtained from BD Biosciences (San Jose, CA). A proline oxidase antibody (T338) was generated in rabbits against a synthetic peptide composed of amino acids 338–353 of the human proline oxidase protein (TGQLEPLLSRFTEEEE). Kidney p53 cDNAs isolated by reverse-transcription (RT)-PCR from primary kidney tissues were cloned into the pcDNA3.1 ECHO expression system (Invitrogen, Inc.). The complete cyclin A1 and proline oxidase cDNAs were PCR-cloned and inserted into the pAdtrack vector [24], which coexpresses green fluorescent protein (GFP) as a normalization control for transfection efficiency. The pAdtrack-proline oxidase and cyclin A1 vectors were used to generate recombinant adenovirus as previously described [24]. The p53 siRNA adenovirus was obtained from Imgenex (San Diego, CA). Recombinant adenoviruses were amplified in 293 cells and isolated by freeze-thawing the cells three times in PBS. Cells were routinely infected with adenoviruses in cell medium at 50 pfu/ml. The cyclin A2 expression vector was a gift from Dr. Fugaku Aoki (University of Tokyo).

Small interfering cyclin A1 RNA. An siRNA specific for cyclin A1 was prepared using the Silencer siRNA Cocktail Kit (Ambion, Inc.). A forward primer (5′-ATGGAGACCGGCTTTCCCGCAAT) and a reverse primer (5′-GACCCCACAGTCAGGGAGTG) containing flanking T7 promoter sequences were used in PCR to generate a 381-bp cyclin A1 DNA fragment. *In vitro* transcription reactions generated RNA from this cDNA, which was subjected to DNase/RNase treatment to digest the template DNA and any single-stranded (ss) RNA remaining in the dsRNA product. The dsRNA was purified on a solid-phase absorption spin column and cleaved with RNase III into short 12–30-bp dsRNAs containing a 5′-PO_4, a 3′-OH, and a 2-nucleotide 3′ overhang; the same structure as siRNA produced *in vivo*. After purification, the siRNA was used in lipofectamine transfections as described below.

Cell culture, transfection, and Western blotting. The 786-0 renal carcinoma, H1299 p53-null non-small cell lung carcinoma, and TOV-21G ovarian adenocarcinoma cell lines were obtained from the American Tissue Type Collection. H1299 cells were propagated in DMEM medium and 786-0 cells were maintained in RPMI medium containing 10% fetal calf serum. TOV-21G cells were grown in a 1:1 mixture of MCDB105 medium (Sigma) and medium 199 (Invitrogen) containing 15% fetal calf serum. To conduct gene transfection of renal and lung carcinoma cells, a T75 flask of confluent cells was incubated for 2 h at 37 °C in 7 ml Optimem medium (Invitrogen, Inc.) containing 10 µg plasmid DNA and 18 µL lipofectamine-2000 (Invitrogen, Inc.). The transfection medium was then replaced with fresh DMEM and the cells incubated for 24–36 h to allow expression of the transfected gene. To normalize for transfection efficiency, pAdtrack, which expresses GFP, was included in the transfection assay where appropriate. Western blotting was performed using the SuperSignal immunodetection system (Pierce Chemical) as previously described [2].

Apoptosis assays. Flow cytometry to quantitate apoptosis was performed on ethanol-fixed and permeabilized cells that were stained with propidium iodide according to a previous published protocol [2]. Apoptotic cells were quantitated in the subG1 DNA content portion of the flow cytometric scans. TUNEL assays were performed and quantitated as previously described [25]. Early apoptosis was detected by immunohistochemistry using a monoclonal antibody (M30) specific for the caspase-cleaved form of cytokeratin 18 [26], according to the manufacturer's specifications (Roche, Inc). Apoptotic cells were also identified by morphological criteria, which included cell blebbing, fragmented and shrunken nuclei, and apoptotic bodies. Annexin-V staining was performed as previously described [27].

The Cell Death Detection ELISA system (Roche Applied Science) is a spectrophotometric enzyme immunoassay for the quantitative in vitro determination of cytoplasm-localized, histone-associated DNA fragments (mono- and oligonucleosomes) generated during apoptotic cell death. Cell lysates were prepared according the manufacturer's specifications and mixed with biotinylated anti-histone antibody in a streptavidin-coated 96-well plate. The biotinylated anti-histone antibody binds to the histone component of free, cytoplasm-localized nucleosomes. The biotinylated antibody-histone immunocomplex was captured by binding to the streptavidin-coated microplate. A peroxidase-conjugated anti-DNA antibody (reacts with ss- and dsDNA in the bound nucleosomes) was added to the lysates in the streptavidin-coated wells. Unbound and nonspecific-binding components in the streptavidin-coated wells were removed by a washing step. Quantitative determination of the amount of bound nucleosomes was determined spectrophotometrically using the peroxidase substrate, 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) at 405 nm.

RT-PCR. Paired normal and carcinoma cDNAs generated from kidney tissues were purchased from Clontech. RT-PCR on non-small cell lung and renal carcinoma cell lines was performed by isolating total RNA using the TRI Reagent extraction solution (Molecular Research Center, Inc.) and generating cDNA from the RNA by using the RT-for-PCR system (Clontech). RT-PCR to analyze cyclin A1 mRNA expression was performed as previously described [2]. Ribosomal protein S9 primers for normalization of PCR were purchased from Clontech, Inc.

Cell synchronization. Cells were synchronized in the late G1/pre-S phase by a double-thymidine treatment. First, subconfluent cell cultures were incubated in complete medium containing 2 mM thymidine for 18 h. Second, the thymidine medium was removed and replaced with complete medium lacking thymidine for 12 h followed by another 18-h incubation in the presence of thymidine. Cells were then harvested for flow cytometry as described above.

Immunohistochemistry. Cells growing on coverslips were washed in PBS, fixed in 2% paraformaldehyde in PBS for 30 min at room temperature, and incubated in 24 mM Tris-base and 192 mM glycine for 5 min at room temperature. The cells were permeabilized by incubating in 0.5% Triton X-100 in PBS for 5 min at room temperature and preblocked by incubating in 0.1% Triton X-100, 1% bovine serum albumin and 1% goat serum overnight at 4 °C. The preblocking solution was removed and primary anti-α-tubulin antibody at 1:200 dilution in preblocking solution added to the cells for 2 h at room temperature. The primary antibody solution was removed and the cells washed four times in PBS. Rhodamine-conjugated anti-rabbit antibody at 1:25 dilution in preblocking solution was added to the cells for 1 h, followed by four washes of the cells in PBS for 20 min. Coverslips containing the stained cells were mounted in Prolong Antifade mounting solution (Molecular Probes, Inc.) and photographed using a Nikon TE2000 ultraviolet microscope.

Cdk1 protein kinase assay. The SignaTECT cdk1 protein kinase assay system (Promega Corp.) was used to measure total cdk1 kinase activity in crude lysates [28], which uses a biotinylated peptide substrate (PKTPKKAKKL) highly specific for the cdk1 kinase.

Results

Altered expression of cyclin A1 in two renal carcinomas is associated with mutated p53. We examined cyclin A1 expression in several primary renal tumors using RT-PCR. cDNAs of normal and tumor tissue sets were obtained commercially (Clontech, Inc.). RT-PCR of mRNA derived from normal renal tissues generated a 0.5-kb product (Fig. 1a, lanes N) that was confirmed as being derived from cyclin A1 mRNA by sequence analysis. One renal carcinoma showed apparent reduced expression (kidney 4, lane T) and another exhibited no expression of cyclin A1 mRNA (kidney 7, lane T), as compared with the apparent noncancerous sections of the same kidneys (lanes N). The control assay (ribosomal protein S9) indicated that quality cDNA was present in the tumor tissue assays. PCR cloning of p53 cDNA revealed that renal carcinomas 4 and 7 expressed mutated p53s [29].

Cyclin A1 is a p53-induced gene in 786-0 renal and H1299 non-small cell lung carcinoma cells. A recombinant p53 adenovirus was used to upregulate p53 in 786-0 renal and H1299 lung carcinoma cells. Forced expression of p53 upregulated the expression of cyclin A1 in 786-0 renal carcinoma cells as indicated by the increased levels of cyclin A1 protein relative to GFP adenovirus-infected cells (Fig. 1b, 786-0 panel, GFP *vs*. p53 lanes). Little or no expression of cyclin A1 was seen in GFP-infected H1299 non-small cell lung carcinoma cells (Fig. 1b, H1299 panel, GFP lane), whereas abundant levels of cyclin A1 appeared in H1299 cells infected with the p53 recombinant adenovirus (Fig. 1b, H1299 panel, p53 lane).

We next investigated whether renal tumor-derived mutant p53s were capable of inducing cyclin A1 in H1299 cells. Sequencing of the p53 cDNAs derived from T4 and T7 renal tumors (analyzed for cyclin A1 expression shown in Fig. 1a) in earlier studies revealed that they expressed mutated p53s [29]. The normal-, T4-, and T7-derived p53 cDNAs were inserted into the ECHO expression vector system as previously described [29]. Transfection of T4 and T7 p53 cDNAs into H1299 cells revealed that both mutant p53s (T4p53, T7p53) were considerably reduced in their ability to upregulate cyclin A1 as compared with the normal kidney-derived p53 (Np53) (Fig. 1c). These two p53 mutants were also defective in their abilities to upregulate endogenous proline oxidase, another p53-induced gene, relative to normal kidney-derived p53 [29]. We concluded that cyclin A1 is a p53-induced gene and its altered expression in two primary renal carcinomas was associated with mutated p53s.

Upregulation of cyclin A1 induces apoptosis in renal carcinoma cells. Since cyclin A1 is a p53-induced gene, it was of interest to determine whether cyclin A1 played any role in apoptosis in renal carcinoma cells. Expression of proline oxidase was also absent in the same renal carcinomas that exhibited altered cyclin A1 expression [29]. We were thus also interested in how cyclin A1 might relate to proline oxidase with respect to apoptosis and the cell cycle. To facilitate our studies, we generated recombinant adenoviruses expressing cyclin A1 or proline oxidase [29] as tools to provide a high efficiency of gene transfection into populations of renal carcinoma cells. To test our recombinant adenoviruses for gene expression, we infected H1299 cells for 24 h with the GFP (used as a control of infection), cyclin A1, or proline oxidase recombinant adenoviruses and performed Western blotting of whole-cell extracts. H1299 cells express very little, if any, cyclin A1 or proline oxidase protein (Fig. 1d, GFP lanes), presumably since these cells lack a functional p53 gene. Upregulation of cyclin A1 and proline oxidase proteins was obtained in H1299 cells infected with cyclin A1 and proline oxidase adenoviruses, respectively (Fig. 1d, Cyc A1 lane and POX lane, respectively). Forced expres-

sion of cyclin A1 in 786-0 cells by infection with the recombinant cyclin A1 adenovirus resulted in increased apoptosis as determined by morphological, TUNEL, annexin-V staining, M30 neoepitope exposure, and flow cytometric assays. Upregulation of cyclin A1 in 786-0 cells increased the exposure of phosphatidylserine on the extracellular cell surface, as detected in flow cytometry by increased binding of fluorescein-conjugated annexin-V relative to GFP-infected cells (Fig. 2a, compare Cyc A1 and GFP panels). Infection of cells with a recombinant proline oxidase adenovirus provided a positive apoptosis control (Fig. 2a, POX panel). 786-0 cells upregulated for cyclin A1 showed increases in the caspase-mediated cleavage of cytokeratin-18 (Fig. 2b), as detected by the exposure of a cytokeratin-18 neo-epitope for the M30 cytodeath monoclonal antibody, an early apoptotic marker [26]. Flow cytometry of ethanol-fixed, propidium iodide-stained cells revealed that cyclin A1 generated considerable alterations in the DNA content profile of flow cytometric scans (Fig. 2c). Increases in apoptotic cells located in the subG1 DNA content of the flow cytometric scans were observed in cyclin A1-induced cells, and quantitation revealed a 27% apoptotic frequency as compared with GFP control cells that exhibited about 9% apoptosis (Fig. 2c). TUNEL assays supported the flow cytometry results by revealing higher amounts of fragmented DNA in cells upregulated for cyclin A1 than in control cells upregulated for GFP (Fig. 2d). More apoptosis was observed in cells upregulated for both cyclin A1 and proline oxidase than in cells upregulated with either protein alone (Fig. 2c, d).

Results from experiments utilizing a cyclin A1 siRNA indicated a role for cyclin A1 in p53-mediated apoptosis. H1299 cells were chosen for these experiments since they do not express p53. Transfection of H1299 cells with a cyclin A1 siRNA prior to infection with the p53 adenovirus resulted in both suppression of the p53-mediated apoptosis and the induction of cyclin A1, as indicated by flow cytometric apoptosis assay (Fig. 3a) and by Western Blot analysis (Fig. 3b), respectively. A control luciferase siRNA (Ambion, Inc.) did not have any effect on p53-induced apoptosis. We concluded from these analyses that upregulation of cyclin A1 can play a role in p53-induced apoptosis in renal and lung carcinoma cells.

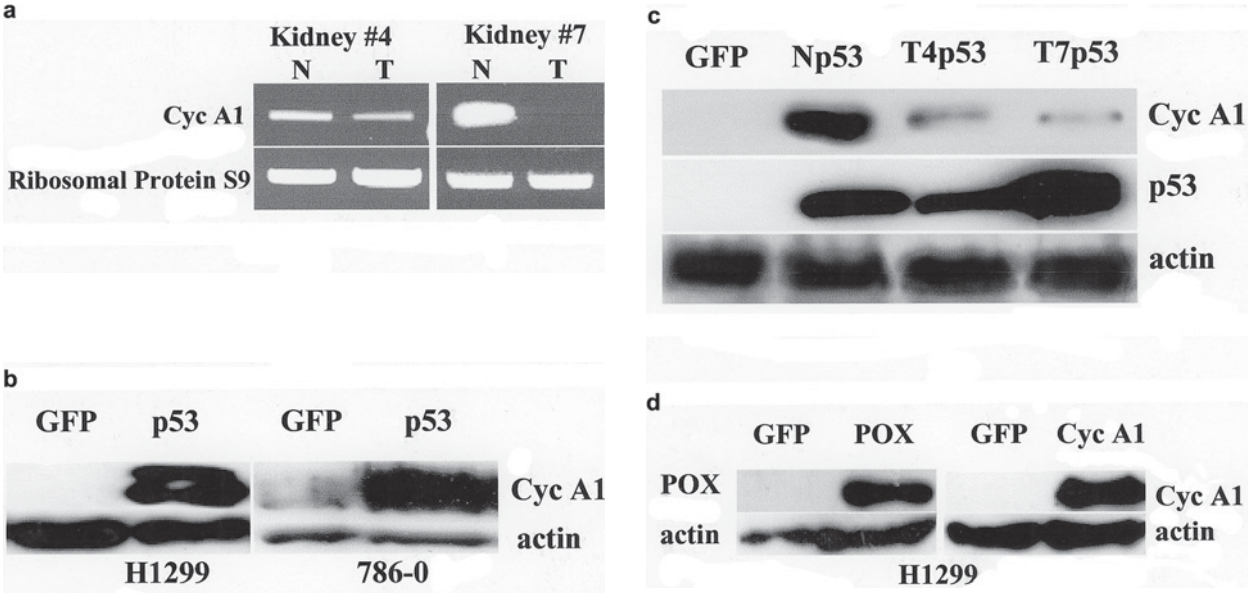


Figure 1. Differential expression of cyclin A1 in tumor cell lines and primary renal carcinoma tissues. (*a*) Expression of cyclin A1 was altered in two primary renal carcinomas that express mutant p53s relative to the comparable normal tissue. RT-PCR to determine cyclin A1 (Cyc A1) mRNA expression was performed on two primary clear-cell type renal carcinomas (T4, T7) and their respective adjacent normal tissue as described under Materials and methods. Ribosomal protein S9 was employed as a normalization control. PCR was conducted for 18, 20, 22, 25, 27, and 30 cycles to insure the exponential nature of the reaction to derive a quantitative result. Shown are the DNA products after 25 cycles of PCR reaction. (*b*) Upregulation of cyclin A1 in 786-0 renal and H1299 lung carcinoma cells after infection with a recombinant p53 adenovirus. Cells were infected for 24 h with 50 pfu/cell GFP or p53 recombinant adenovirus. Expression of cyclin A1 (Cyc A1) and actin (protein loading control) was examined by Western blotting. (*c*) Renal carcinoma-derived mutated p53s were deficient in their ability to upregulate cyclin A1. The p53 cDNAs derived from renal carcinomas T4 (T4p53) and T7 (T7p53) that showed reduced cyclin A1 expression in (*a*) were cloned into ECHO expression plasmids and sequenced, which identified each as encoding a mutated p53 [29]. Forced upregulation of normal p53 (Np53), T4p53, and T7p53 was conducted by transfection of ECHO plasmids expressing each of the p53 cDNAs. A GFP ECHO expression vector was used as a negative control. Expression of p53, cyclin A1 (Cyc A1), and actin (protein loading control) was examined by Western blotting. (*d*) Forced upregulation of proline oxidase and cyclin A1 proteins by recombinant adenoviruses in H1299 cells. Proline oxidase was used in some of our experiments as a p53-induced proapoptotic control gene. Cells were infected with GFP, proline oxidase (POX), and cyclin A1 (Cyc A1) recombinant adenoviruses as described above for p53 in (*b*). Expression of proteins was determined by Western blot using a peptide POX antibody [29] and commercially derived antibodies for cyclin A1 and actin.

Cyclin A1 but not cyclin A2 induces apoptosis. To determine whether other members of the cyclin A family could induce apoptosis, we transfected H1299 cells with plasmids expressing either cyclin A1 or cyclin A2. At 24 h post transfection, apoptosis was measured by a cell death ELISA assay, which quantitates fragmented nucleosomal DNA generated in cells undergoing apoptosis. Figure 3d shows a Western blot of cyclin A1 (CycA1) and cyclin A2 (CycA2) in transfected cells. Only cells upregulated for cyclin A1 showed significant increases in apoptosis (Fig. 3c, CycA1), indicating a cyclin A1-specific effect on apoptosis.

Cyclin A1 induces G2/M arrest and promotes DNA polyploidy. A number of studies have implicated cyclin A1 in regulation of the cell cycle. To determine any effect of cyclin A1 on the cell cycle of human carcinoma cells, we studied the progression of synchronized H1299 cells upregulated for cyclin A1 through the cell cycle. Based on our above experimental results, we were also interested in potential cooperative effects of cyclin A1 and proline oxidase on the cell cycle. To conduct our cell-cycle studies, we infected H1299 cells with an equal Multiplicity of infection (50 pfu/cell) of GFP, proline oxidase, cyclin A1, or a combination of cyclin A1 and proline oxidase (cyclin A1 + POX) adenoviruses, followed by synchronization of the cells at late G1/early S with a double-thymidine (2 mM) block. The cells were released from the thymidine block and harvested for flow cytometry and analysis of DNA content (by propidium iodide staining) at different time points (0–12 h) following the release. To prepare the cells for flow cytometry, we fixed the cells first with 2% paraformaldehyde before fixation in 70% ethanol to prevent any fragmented DNA in apoptotic cells from washing out during the processing procedure for flow cytometry. Thus, a subG1 DNA content for apoptotic cells is not apparent in this flow cytometric experiment. Figure 4 shows the flow cytometric scans of cells that were synchronized by a thymidine

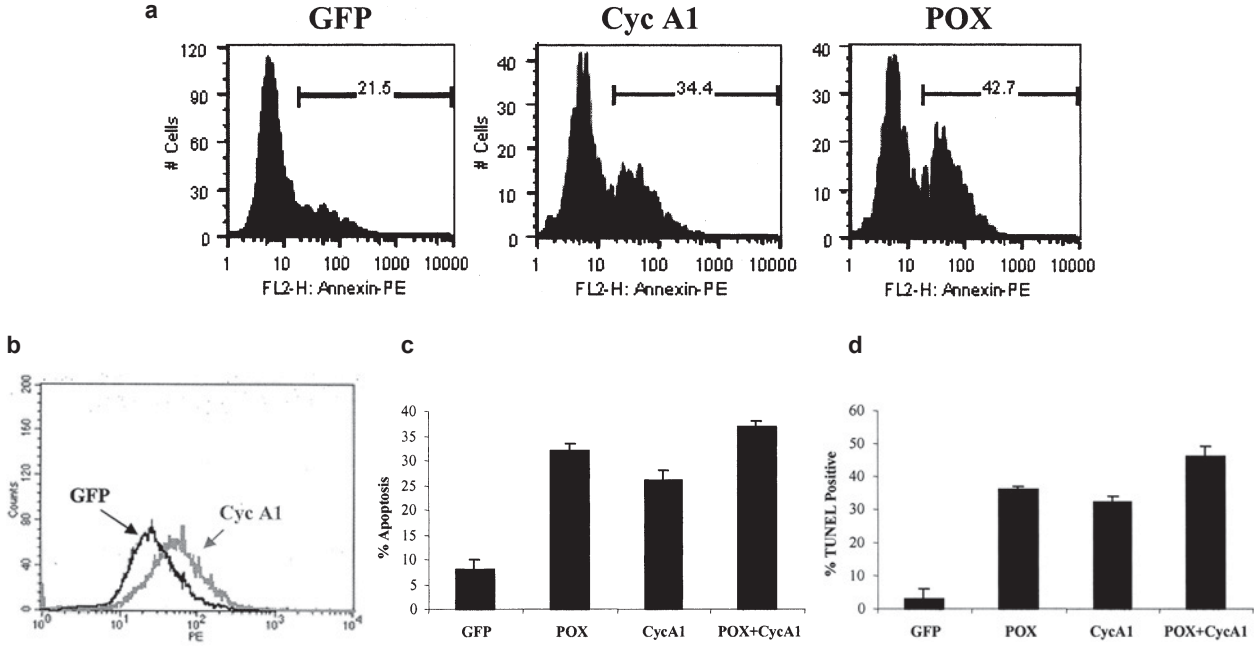


Figure 2. Cyclin A1 induces apoptosis in 786-0 renal carcinoma cells. (*a*) Upregulation of cyclin A1 induced the externalization of phosphatidylserine in 786-0 renal carcinoma cells. 786-0 cells were infected with GFP, cyclin A1 (Cyc A1), or proline oxidase (POX) adenoviruses for 24 h. Cells were then stained with phycoerythrin-conjugated annexin-V and analyzed by flow cytometry as described under Materials and methods. Upregulation of cyclin A1 increased the exposure of phosphatidylserine on the cell surface of cells, as indicated by the increase in phycoerythrin fluorescence of cyclin A1-induced cells (Cyc A1). Infection of cells with a proline oxidase adenovirus (POX) provided a positive apoptosis control. (*b*) Cyclin A1 induced caspase-cleaved forms of cytokeratin-18 in 786-0 cells. 786-0 cells were infected with GFP or cyclin A1 (Cyc A1) adenovirus for 24 h and processed as described in Materials and methods to analyze the expression of a neoepitope that is exposed only in caspase-cleaved forms of cytokeratin-18. The early apoptotic neoepitope marker of cytokeratin-18 was detected using the M30 monoclonal antibody, a phycoerythrin-conjugated anti-mouse antibody, and flow cytometry. Upregulation of cyclin A1 generated increases in the M30 neoepitope of cytokeratin-18 in 786-0 cells, as detected by the shift to the right (increase) in PE fluorescence, indicating increases in caspase activity. (*c*) Quantitation of apoptosis in GFP-, proline oxidase-, and cyclin A1-induced 786-0 cells by flow cytometry of propidium iodide-stained cells. Cells were infected for 24 h with GFP, cyclin A1 (CycA1), proline oxidase (POX), or a combination of proline oxidase and cyclin A1 (POX+CycA1) adenoviruses, fixed in ethanol, and subjected to flow cytometry. Quantitation of sub G1 DNA content (apoptotic cells) in flow cytometric scans is shown. (*d*) Quantitation of apoptosis in 786-0 cells by TUNEL flow cytometry. 786-0 cells were infected with GFP, cyclin A1 (CycA1), proline oxidase (POX), or a combination of proline oxidase and cyclin A1 (POX+CycA1) adenoviruses for 30 h and processed for TUNEL and flow cytometry as described under Materials and methods. The data are quantitated as the percentage of apoptotic cells derived from the incorporated PE-labeled BrdU antibody fluorescence that was higher than 10^2 fluorescence intensity units in flow cytometric scans.

block and then prepared for flow cytometry at 0, 2, 6, 8, and 12 h after release from the thymidine block. Overlays of the flow cytometric scans of unsynchronized GFP cells (---) and synchronized cells upregulated for GFP (—), cyclin A1 (—), or proline oxidase (—) are shown in Fig. 4a. The locations of the G1, S, and G2 phase populations are designated by the unsynchronized cell scan (---). GFP-only-expressing cells (—) moved from late G1/early S into late S/early G2 approximately 6 h after release from the thymidine block and then returned to G1 DNA content after undergoing division at 12 h. Cells upregulated for cyclin A1 (—) or proline oxidase (—) progressed from late G1/early S into G2 and remained there throughout the 12-h release period, with substantial numbers of cells becoming progressively higher in DNA content (polyploidy) than the 4N DNA content of G2 cells, which may be due to these cells undergoing the process of endoreduplication. This experiment indicates that cyclin A1 or proline oxidase can suppress the growth of renal carcinoma cells by generating a G2/M arrest. Co-upregulation of both proteins resulted in a very efficient G2/M cell cycle arrest and generation of polyploid cells (Fig. 4b, solid black line). Apoptosis then occurred after the cells became polyploid; the 12-h release time point is equivalent to 24 h post infection with proline oxidase or cyclin A1 adenovirus, a time when extensive morphological signs of apoptosis become evident in the cell culture, as exemplified by rounded, floating and shrunken cells, many intracellular vacuoles, condensation of the nucleus, and blebbing of the plasma membrane.

Cyclin A1 and p53 induce mitotic catastrophe in H1299 cells. Cells undergoing mitotic catastrophe are large and flat, and can contain multiple micronuclei with uncondensed chromosomes [30]. Mitotic catastrophe is thus easily distinguished from apoptotic cells, which exhibit a rounded and shrunken morphology and irregular, fragmented nuclei with condensed chromatin [30]. Cells in mitotic catastrophe can undergo endocycles of DNA replication, which can lead to polyploidy and eventual apoptosis [31], and might explain the emergence of polyploid cells upregulated for cyclin A1 and proline oxidase. To determine whether cells upregulated for cyclin A1 and proline oxidase were undergoing mitotic catastrophe, we transfected thymidine-synchronized H1299 cells with GFP, p53, cyclin A1, proline oxidase, or a combination of cyclin A1 and proline oxidase adenoviruses. The cells were released from the second thymidine block, and 12 h later prepared for immunohistochemistry using an antibody specific for α-tubulin to visualize the cytoskeleton and spindle apparatus. The recombinant adenoviruses were constructed to co-express GFP along with the cyclin A1 or proline oxidase transgene, thus allowing convenient visualization of cells expressing cyclin A1 or proline oxidase. A virus concentration of 0.1 pfu/cell was employed to compare side-by-side uninfected and transgene-infected

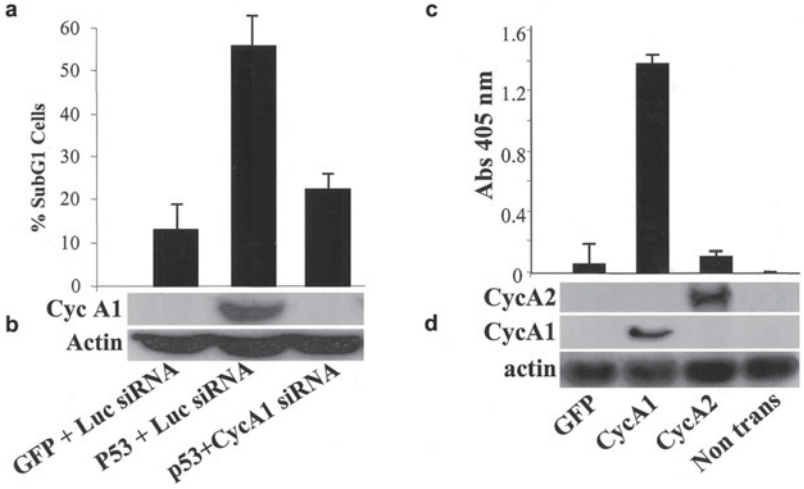


Figure 3. A cyclin A1 siRNA suppressed p53-mediated induction of cyclin A1 and apoptosis. Upregulation of cyclin A2 did not induce apoptosis. (*a*) Suppression of cyclin A1 expression reduced p53-induced apoptosis. H1299 cells were chosen for these experiments since they do not express p53. Cells were first transfected for 4 h with cyclin A1 siRNA (CycA1 siRNA) or a luciferase siRNA (Luc siRNA) using lipofectamine-2000 followed by infection with GFP or p53 adenovirus for 24 h. Cells infected with the p53 adenovirus underwent increased apoptosis relative to GFP-infected cells in the presence of the luciferase siRNA control. The presence of cyclin A1 siRNA in p53-infected cells resulted in suppression of p53-induced apoptosis. (*b*) Western blot of cyclin A1 expression in the transfected cells shown in (*a*). The induction of cyclin A1 protein expression by p53 was suppressed by the cyclin A1 siRNA. Whole-cell lysates prepared from GFP- (lane 1), p53+luciferase siRNA- (lane 2), and p53+cyclin A1 siRNA- (lane 3) transfected cells were subjected to Western blotting to determine cyclin A1 expression. The cyclin A1 siRNA effectively suppressed p53-induced upregulation of cyclin A1 protein. (*c*) Apoptosis is induced by cyclin A1 but not cyclin A2. Cell death ELISA assay of nontransfected control cells (Non trans) and cells transfected with GFP, cyclin A1 (CycA1), cyclin A1 (CycA2). H1299 cells were transfected with a cyclin A1 or cyclin A2 expression plasmid and evaluated for protein expression and apoptosis 36 h later. (*d*) Western blot of transfected cells that were analyzed for apoptosis in (*c*). Whole-cell lysates were probed with either anti-cyclin A2 (CycA2), anti-cyclin A1 (CycA1), or anti-actin (actin). Only cells upregulated for cyclin A1 showed marked increases in apoptosis.

cells. Some cyclin A1- and proline oxidase-induced cells exhibited a much larger flattened morphology containing altered cytoskeletal structures and multiple micronuclei, which are hallmark characteristics of mitotic catastrophe (Fig. 5, red arrows), as well as numerous shrunken, rounded cells containing condensed nuclei characteristic of apoptosis (Fig. 5, white arrows). Very few cells exhibiting these morphologies were observed in GFP-control infected cultures or in nontransfected cells (Fig. 5). H1299 cells upregulated for p53 also showed morphological characteristics of mitotic catastrophe and apoptosis (Fig. 5).

Cyclin A1 upregulates cdk1 protein and kinase activity. Cyclin A1 and proline oxidase might mediate a G2/M arrest by affecting the expression and activity of the cyclin B1/cdk1 pathway, which is a major cell-cycle checkpoint in progression of cells through mitosis [32] and is downregulated by p53 [33]. Moreover, upregulation and activation of cdk1 plays a role in mitotic catastrophe in some types of cells [34, 35]. We thus examined the expression of cyclin B1/cdk1 protein and kinase activity in thymidine-synchronized H1299 cells upregulated for cyclin A1 or proline oxidase. Cells were treated with 2 mM thymidine and infected with cyclin A1 or proline oxidase adenovirus or both. Cells were then released from the thymidine block by removal of thymidine and analyzed for expression of cyclin B1/cdk1 proteins and kinase activity at 0 and 7 h after release from the block. After 7 h of release from the thymidine block, the cells would have progressed into G2 as determined from the cell-cycle studies shown in Fig. 4. Thymidine-blocked cells upregulated for cyclin A1 or proline oxidase expressed considerably more cdk1 protein than control cells induced with GFP (Fig. 6b, 0 h). More cdk1 kinase activities were also observed in both thymidine-blocked cyclin A1- and proline oxidase-induced cells than in blocked GFP-induced cells (Fig. 6a, 0 h). Further increases in cdk1 protein expression were observed in all cells after 7 h of release from

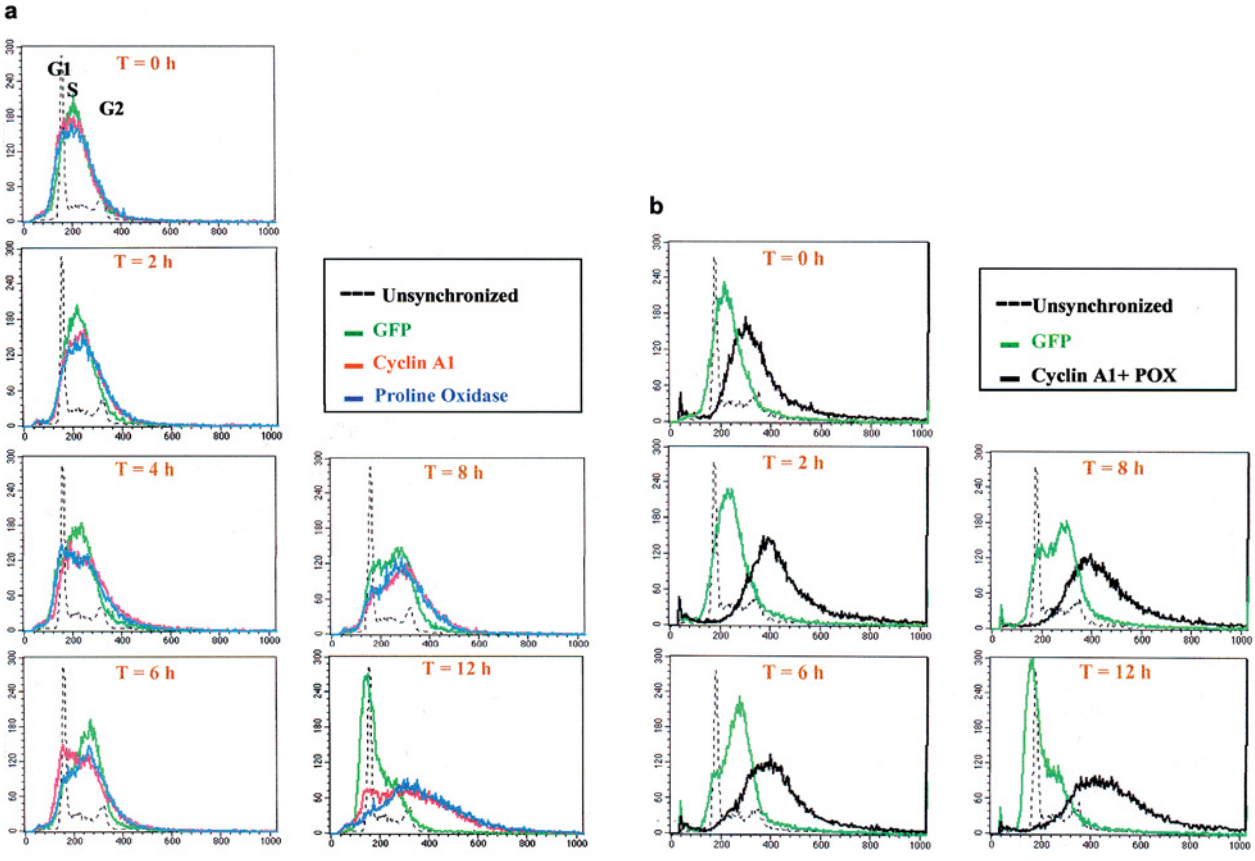


Figure 4. Cell-cycle analysis of H1299 cells upregulated for GFP, proline oxidase, cyclin A1, or a combination of cyclin A1 and proline oxidase. H1299 cells were synchronized at late G1/early S by a double-thymidine block as described under Materials and methods. Cells were upregulated for transgenes by recombinant adenovirus infection during the second 18-h treatment with thymidine. Cells were then released from the second thymidine block by replacing the thymidine- and virus-containing medium with medium lacking thymidine and adenovirus. Cells were harvested at different times after release from the thymidine block for analyses of DNA content by propidium iodide staining and flow cytometry. (*a*) Flow cytometric analyses of GFP-, cyclin A1-, or proline oxidase-induced synchronized cells for DNA content at 0, 2, 4, 6, 8, and 12 h after release from the thymidine block. (*b*) Flow cytometric analyses of GFP-control cells and cells upregulated for both cyclin A1 and proline oxidase (Cyclin A1+POX) after 0, 2, 6, 8, and 12 h of release from the thymidine block. Cells upregulated for cyclin A1 or proline oxidase showed a delay in the cell cycle resulting in an accumulation of G2 arrested cells with progressing polyploidy. Upregulation of a combination of cyclin A1 and proline oxidase resulted in a very efficient G2 arrest and polyploidy.

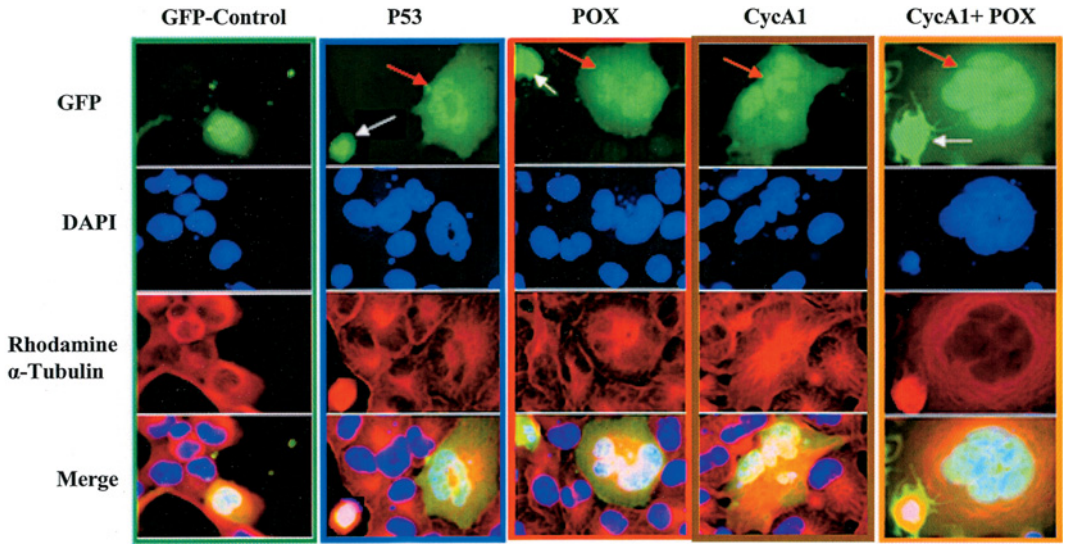


Figure 5. H1299 cells upregulated for cyclin A1 and proline oxidase show morphological signs of apoptosis and mitotic catastrophe. Cells were synchronized by a thymidine block and infected with GFP, p53, cyclin A1 (CycA1), proline oxidase (POX) adenoviruses or a combination of cyclin A1 and proline oxidase adenoviruses (CycA1+POX). At 12 h after release from the thymidine block, cells were fixed in paraformaldehyde and subjected to immunofluorescence using a monoclonal antibody for α-tubulin. Cells upregulated for p53, proline oxidase, or cyclin A1 were identified by GFP fluorescence, which is coexpressed from the pAdtrack vector used to express the proline oxidase and cyclin A1 transgenes. A rhodamine-conjugated anti-mouse antibody was used to detect α-tubulin immunoreactivity. Cells were counterstained with DAPI to visualize the nuclei. Many cells upregulated for p53, cyclin A1, or proline oxidase showed a large flattened morphology and multiple aberrant nuclei characteristic of mitotic catastrophe (red arrows). Apoptotic cells, in contrast, were characterized by a shrunken, rounded morphology with condensed chromatin and nuclei (white arrows).

the thymidine block (G2 stage; Fig. 6b, 7 h) than in cells at the G1/early S phase (0 h). Remarkably more cdk1 protein was observed in G2 cells upregulated for cyclin A1 or proline oxidase than in G2 control GFP cells. From these data, we concluded that both cyclin A1 and proline oxidase can upregulate cdk1 protein and kinase activity. However, cdk1 kinase activities in cyclin A1 and proline oxidase-induced cells were much lower than what would be expected from the increases in expression of cdk1 protein, suggesting that a significant population of the upregulated cdk1 protein was not active for protein kinase activity.

Cyclin A1 is upregulated in etoposide-treated TOV-21G ovarian carcinoma cells. Etoposide is a genotoxic agent that is known to upregulate p53 and activate its DNA binding activity. Concentrations of etoposide ranging from 0 to 141 µM induced considerable apoptosis in TOV-21G cells after 24 h [36]. P53 and two p53-induced genes, $p21^{waf1/cip1}$ and proline oxidase, were upregulated in TOV-21G cells in response to etoposide treatments [36]. TOV-21G cells express a normal p53 [37]. The expression of cyclin A1 was thus examined in etoposide-treated TOV-21G cells. As shown in Fig. 7, both p53 and cyclin A1 expression increased in correlation with the increases in the concentration of etoposide, a result consistent with a role for cyclin A1 as a p53-induced gene that plays a role in p53-dependent apoptosis and DNA repair [3]. In contrast,

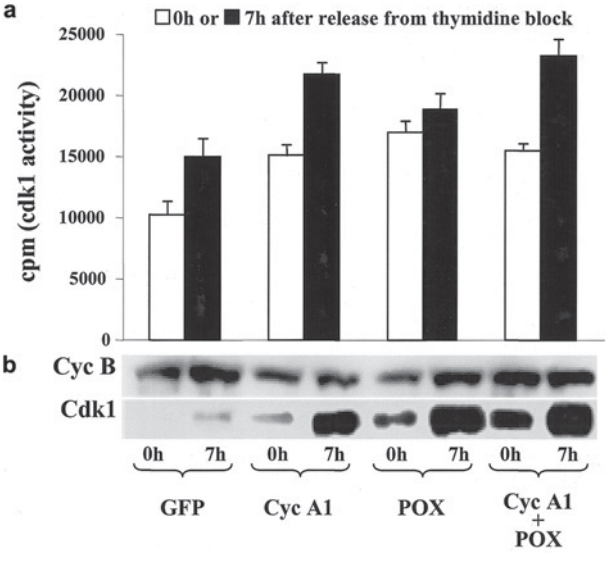


Figure 6. Cyclin B/cdk1 protein and kinase activity in H1299 cells upregulated for cyclin A1 and proline oxidase. Cells were transfected with GFP, cyclin A1 (Cyc A1), proline oxidase (POX), or Cyc A1+POX for 12 h and then synchronized in late G1/early S by a thymidine block. Whole-cell lysates were then prepared for Western blotting or cdk1 kinase assays as described under Materials and methods at 0 (T0) or 7 h after release from the thymidine block (T7), when the cells would be in late S/early G2 as indicated in the flow cytometric results shown in Fig. 4. (*a*) cdk1 protein kinase activity. (*b*) Western blot of cyclin B (Cyc B) and cdk1 proteins in cells assayed in (*a*). Markedly more cdk1 protein and kinase activities were present in cells upregulated for cyclin A1 and proline oxidase than in control GFP cells.

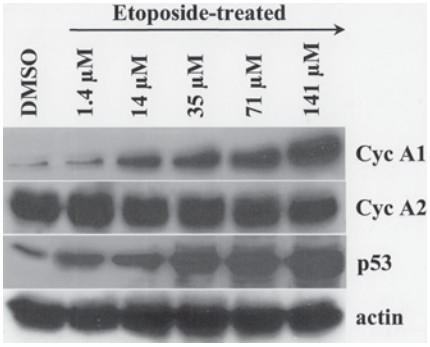


Figure 7. Cyclin A1 but not cyclin A2 is upregulated in TOV-21G ovarian carcinoma cells undergoing p53-dependent apoptosis induced by etoposide. TOV-21G cells were treated with 0–141 µM etoposide for 24 h and then subjected to Western blotting to determine cyclin A1 (Cyc A1), cyclin A2 (CycA 2), and p53 protein levels. Actin was employed as a protein loading control. Cyclin A1 but not cyclin A2 levels increased in correlation with increases in p53 expression in etoposide-treated cells.

slight decreases in cyclin A2 expression were observed between control and etoposide-treated cells (Fig. 7).

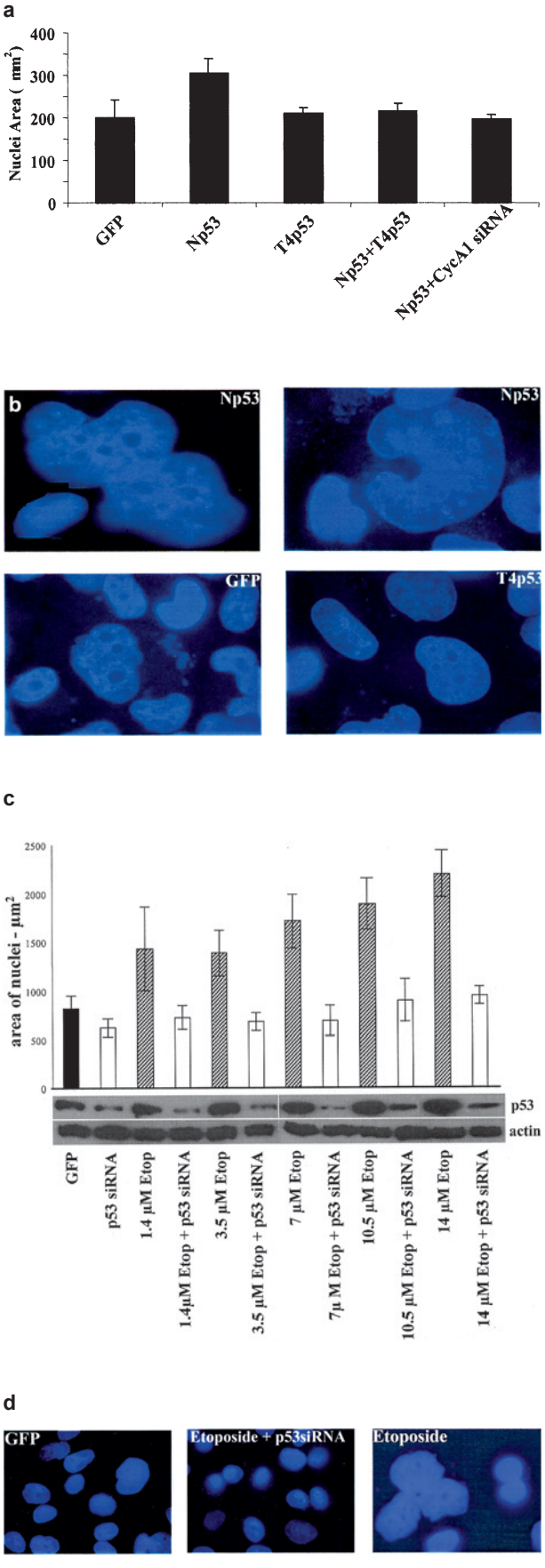
Cyclin A1 plays a role in p53-mediated mitotic catastrophe in H1299 cells. Morphological characteristics of mitotic catastrophe were observed in numerous H1299 cells upregulated for p53. Representative images of DAPI-stained nuclei of GFP- (control) and p53-induced (Np53) cells are shown in Fig. 8b. In contrast to GFP-induced cells, numerous p53-expressing (Np53) cells exhibited multinucleated morphology, indicative of mitotic catastrophe. Mitotic catastrophe was specific for the normal p53, since cells expressing the T4p53 mutant, which is defective in upregulating cyclin A1, did not exhibit the enlarged, multinucleated phenotype. Since mitotic catastrophe was characterized by enlarged multinuclei, the average area of nuclei in fifty microscopic fields was compiled as a method to quantitate mitotic catastrophe (Fig. 8a). Quantitation of nuclear size revealed an average increased nuclear size in normal p53 (Np53) but not in T4p53 mutant cells relative to GFP cells. The T4p53 is a dominant-negative inhibitor of Np53 [36] and it effectively suppressed the emergence of polyploid cells induced by Np53. The cyclin A1 siRNA also effectively suppressed increases in nuclear size induced by Np53 (Fig. 8a).

Cyclin A1 mediates in part the p53-dependent etoposide-induced mitotic catastrophe in TOV-21G ovarian carcinoma cells. We observed many TOV-21G cells undergoing mitotic catastrophe in the presence of etoposide (Fig. 8d, right panel). To determine the role of p53 in etoposide-induced mitotic catastrophe, TOV-21G cells were treated with varying doses of etoposide and the GFP control or p53 siRNA adenovirus for 36 h. Nuclear sizes in the cell populations increased along with increases in the expression of p53 in cells treated with etoposide (Fig. 8c). The p53 siRNA adenovirus effectively suppressed the upregulation of p53 by etoposide (Fig. 8c). Little or no increases in nuclear size or p53 expression were observed in etoposide-treated cells infected with the p53 siRNA adenovirus (Fig. 8c). In the presence of etoposide, many GFP-expressing control cells exhibited a large flattened morphology and irregular multinuclei, which are defining characteristics of mitotic catastrophe (Fig. 8d, right panel). Very few cells exhibiting these morphologies were observed in etoposide-treated cells infected with the p53 siRNA (Fig. 8d, middle panel) or in cells infected with the GFP adenovirus (Fig. 8d, left panel). Transfection of cyclin A1 siRNA into TOV-21G cells reduced the ability of etoposide to upregulate cyclin A1 levels and to induce mitotic catastrophe (Fig. 9), implicating a role for cyclin A1 in etoposide-induced mitotic catastrophe.

Discussion

DNA damage leads to the stabilization and activation of p53, which then transcriptionally induces several BH3 domain-only proteins that function upstream of BAX/BAK to induce apoptosis, including the PUMA (p53 upregulated modulator of apoptosis) and Noxa (Latin for 'harm') genes [38, 39]. Another group of proapoptotic genes induced by p53 (the PIGs) mediate apoptosis through redox pathways. The proline oxidase gene is one member of the PIG class (PIG6) that encodes a mitochondrion-localized protein that oxidizes proline and generates reactive oxygen species [40]. We report here a new p53-induced apoptotic pathway involving the cyclin A1 gene. We first identified cyclin A1 as a p53-regulated gene by cDNA expression profiling of p53-sensitive and -resistant tumor cells [2]. We present several lines of evidence in this manuscript that strongly implicate cyclin A1 in p53-mediated apoptosis. First, the forced upregulation of cyclin A1 resulted in apoptosis. Second, p53-induced upregulation of cyclin A1 protein and apoptosis were suppressed by cyclin A1 siRNA. Third, apoptosis induced by the cyclin A family was specific to the A1 member, since upregulation of cyclin A2 did not induce apoptosis. Fourth, cyclin A1 siRNA suppressed p53-dependent apoptosis induced by etoposide. Co-upregulation of both proline oxidase and cyclin A1 was somewhat more effective in mediating apoptosis than either protein alone. We plan to investigate whether other p53 proapoptotic genes such as PUMA and Noxa can also cooperate with cyclin A1 to induce apoptosis.

Cyclin A1 was also capable of inducing mitotic catastrophe in lung, ovarian, and renal carcinoma cells. Mitotic catastrophe is a form of cell death that results from aberrant mitosis, leading to the formation of large nonviable cells with numerous micronuclei containing uncondensed



chromosomes [30]. Mitotic catastrophe is a mechanism widely found in development and can be a sign of abnormality in tumor progression [41]. Cells undergoing mitotic catastrophe are easily distinguishable from apoptotic cells based on morphology, and would probably not be detected using TUNEL or flow cytometry, since cells that die through mitotic catastrophe usually do not show DNA ladder formation or DNA breaks [30]. We observed both mitotic catastrophe and apoptosis occurring in H1299, TOV-21G, and 786-0 cells after ectopic expression of p53. Our p53 and cyclin A1 siRNA experiments suggested that p53 can induce mitotic catastrophe, at least in part, through the upregulation of cyclin A1. Perhaps cyclin A1-induced mitotic catastrophe is a process whereby p53 eliminates those cells that have developed a resistance to apoptosis. For instance, expression of anti-apoptotic proteins such as Bcl-2 and MDR1 prevents apoptosis induced by chemotherapeutic agents or ionizing radiation, but does not improve clonogenic survival because the cells undergo mitotic catastrophe [30]. Cyclin A1-induced mitotic catastrophe may be a tumor suppression checkpoint in those cells that have developed an inherent resistance to apopto-

Figure 8. Cyclin A1 plays a role in p53-induced mitotic catastrophe in TOV-21G cells. Cells undergoing mitotic catastrophe exhibit a large flattened morphology, large multinuclei, and polyploidy. Since cells undergoing mitotic catastrophe exhibit enlarged multinuclei, we quantitated mitotic catastrophe based on nuclear area. Imaging and nuclear area estimates were performed with a Nikon TE2000 ultraviolet microscope using the MetaMorph Imaging System software (Molecular Devices Corporation, Chicago, IL). Morphometric measurements were taken on eight randomly selected fields at 60 × magnification. (*a*) Upregulation of p53 increased nuclear size. Upregulation of normal p53 resulted in the formation of large, multinuclei, which were quantitated based on nuclear size. Increased nuclear size was induced in normal p53-transfected (Np53) cells relative to GFP control cells. A dominant-negative mutant p53 (T4p53) did not induce increases in nuclear size and prevented Np53 from doing so. A cyclin A1 siRNA (CycA1 siRNA), which suppresses cyclin A1 expression (Fig. 3b), prevented Np53 from inducing increases in nuclear size. (*b*) Upregulation of p53 induced mitotic catastrophe. Induction of normal p53 (Np53) in H1299 cells resulted in the appearance of numerous cells exhibiting the multinucleated morphology of mitotic catastrophe (top panels). Very few of these types of cells were observed in cells upregulated for GFP or the T4p53 mutant (lower panels). (*c*) p53 is involved in etoposide-induced mitotic catastrophe in TOV cells. Cells were treated with different doses of etoposide ranging from 1.4 to 14 µM during infection with a GFP control or p53 siRNA adenovirus for 36 h. The p53 siRNA effectively suppressed the induction of p53 by all concentrations of etoposide. The nuclear size of cells treated with etoposide increased along with increases in the expression of p53. Increases in nuclear size and p53 expression did not occur in cells treated with the p53 siRNA adenovirus or in cells treated with GFP adenovirus alone. (*d*) Nuclear morphology of cells in the presence of etoposide. Many GFP control cells in the presence of etoposide exhibited large irregular multinuclei, indicative of mitotic catastrophe (far right panel). Very few cells exhibiting this nuclear morphology were observed in etoposide-treated cells expressing p53 siRNA (middle panel) or in cells expressing GFP alone (far left panel).

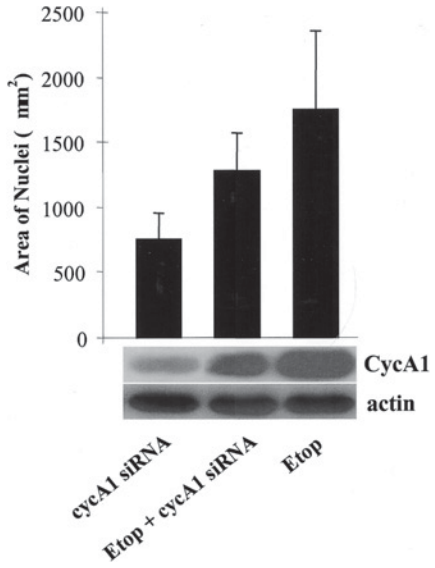


Figure 9. Cyclin A1 plays a role in etoposide-mediated mitotic catastrophe of TOV-21G cells. Cells grown on glass coverslips were transfected with either cyclin A1 siRNA (cycA1 siRNA), 14 μM etoposide +cyclin A1 siRNA, or 14 μM etoposide (Etop) only for 24 h. Cells were fixed and stained with the DAPI nuclear stain as described under Materials and methods. Imaging and nuclear area estimates were performed with a Nikon TE2000 ultraviolet microscope using the MetaMorph Imaging System software (Molecular Devices Corporation). Morphometric measurements were taken on eight randomly selected fields at 60 × magnification. Increases in nuclear size correlated positively with the level of expression of cyclin A1.

sis, thus providing a second layer of protection against the emergence of cancerous cells.

We observed upregulation of cdk1 protein and increased cdk1 protein kinase activity in cyclin A1-induced cells. It has been proposed that mitotic catastrophe results from premature induction of mitosis before the completion of S or G2 [42]. Inappropriate upregulation of cyclin A1 and the resulting increases in cdk1 that we observed might cause a premature induction of mitosis and, along with continued upregulation of cdk1, could interfere with the completion of mitosis, thus leading to polyploidy and mitotic catastrophe. Indeed, several lines of evidence noted below imply that cyclin A1 might regulate the G2 phase of the cell cycle by influencing the activity of cdk1. Moreover, cdk1 can actually induce apoptosis and mitotic catastrophe in some instances. First, cyclin A1 is known to regulate the activity of cdks. For instance, cyclin A1-dependent kinase complexes contain both cdk1 and cdk2 components [43] and cyclin A1 plays an essential role in initiating the activation of cyclin B1/cdk1 kinase at the meiotic G2/M transition of male germ cells [10]. The activation of the cyclin B/cdk1 complex drives progression from G2 to M phase, and its activity must be sustained from the prophase to the metaphase [32]. The sudden destruction of the cyclin B/cdk1 activity is required for subsequent entry into the anaphase. Second, ectopic overexpression of cyclin B and cdk1 has been reported to lead to mitotic catastrophe [44]. Third, activation of the cdk1 pathway has been associated with some cases of apoptosis. For instance, microtubule damage induced by paclitaxel inhibits proteasome-dependent degradation of cyclin B1, resulting in a sustained activation of cyclinB1/cdk1 kinase and a cell-cycle arrest in mitosis, followed by apoptosis [45]. Fourth, taxol-induced cell death is associated with increases in the activity of cdk1 in several breast cancer cell lines, and chemical inhibition of cdk1 or transfection with a dominant-negative cdk1 blocks taxol-induced cell death [46]. Fifth, cdk1 has also been reported to activate the apoptotic pathway by inducing mitochondrial membrane permeabilization through the phosphorylation of Bcl-2 family proteins [47]. Finally, a direct interaction between cyclin A1 and cdk1 was found in retinoic acid-treated leukemic cells but not in untreated cells [48]. Based on these reports and our data, we propose that the increased cdk1 protein expression and kinase activities that we observed in cyclin A1-induced cells can lead to mitotic catastrophe, which is followed by apoptosis. Apoptosis does not precede mitotic catastrophe but apoptosis frequently follows mitotic catastrophe [30].

The p53 protein regulates multiple cell cycle checkpoints that control the mammalian response to DNA damage. When transcriptionally upregulated by p53, the $p21^{WAF1/CIP1}$ protein has a decisive role in cell-cycle arrest [49]. Forced expression of p21 can result in G1-, G2- [50], or S-phase arrest [51]. Regulation of G2 by p53 can also be mediated through its ability to downregulate the M-phase promoting factor (MPF), a universal cell-cycle regulatory complex, which consists of two proteins, cyclin B and cdk1 [52]. The degradation of the cyclin B subunit of protein kinase Cdk1/cyclin B is required for inactivation of the kinase and exit from mitosis. In response to DNA damage, both cdk1 and cyclin B1 expression are repressed by p53 at the transcriptional level resulting in a G2 arrest [53, 54]. We provide evidence in this manuscript for yet another pathway whereby p53 can induce an effective G2 arrest, which is mediated through the upregulation of cyclin A1 and proline oxidase.

Altered expression of both cyclin A1 and proline oxidase could play a role in the development of renal carcinoma, since the expression of both proteins was either absent or reduced in two primary renal carcinomas that expressed mutated p53s. A tumor suppressor role for cyclin A1 is suggested by an inverse relationship between methylation of the cyclin A1 gene and p53 mutational status [20]. Moreover, forced expression of cyclin A1 resulted in robust induction of wild-type p53 in HNSCC cell lines [20]. Hypermethylation of the cyclin A1 gene has also been observed in a significant number of hepatocellular carcinomas [55]. Furthermore, specific induction of cyclin A1 expression and promoter activity after UV and γ-irradiation was mediated by p53 [3]. Finally, cyclin A1 was

the only member of the cyclin A family to be induced by γ-irradiation and p53, and cyclin A1-deficient MEF cells, which expressed cyclin A2, were deficient in DNA ds base repair [3]. The reduced or absent expression of cyclin A1 and proline oxidase by mutation of p53 or hypermethylation of the respective gene promoter might result in tumor cells gaining resistance to mitotic catastrophe and apoptosis, thus providing those cells a mechanism to survive exposures to irradiation and chemotherapeutic drugs.

In contrast to the findings discussed above that suggest its potential role as a tumor suppressor gene, cyclin A1 is believed to play a role in the pathogenesis of myeloid leukemia, since it is highly expressed in leukemias of myeloid origin [12]. Furthermore, cyclin A1 expression increased upon induction of myeloid differentiation [12]. The highest frequency of cyclin A1 overexpression was observed in acute myelocytic leukemias, especially those that were at the promyelocyte (M3) and myeloblast (M2) stages of development [12]. Overexpression of murine cyclin A1 in transgenic mice leads to abnormal myelopoiesis in the first months after birth as well as to the development of myeloid leukemia at a low frequency, indicating that cyclin A1 alone is not sufficient to induce transformation but contributes to leukemogenesis [17]. Interestingly, the localization of cyclin A1 in normal hematopoietic cells is nuclear, whereas in leukemic cells from AML patients and cell lines, it is predominantly cytoplasmic [48]. Our work and the other studies discussed above suggest that upregulation of cyclin A1 can have either tumor-promoting or tumor-suppressing activities depending on the cellular context and tissue. Clearly, more study is required to clarify the functional role of cyclin A1 in tumorigenesis.

The data we present in this manuscript lead us to propose that upregulation of cyclin A1 is a novel pathway whereby p53 can mediate a G2/M cell-cycle arrest, potentially providing an additional DNA repair checkpoint in certain types of cells such as renal, ovarian, and lung carcinoma cells. Both cyclin A1 and proline oxidase are induced in etoposide-treated cells, and may work together in facilitating efficient DNA repair. The proline oxidation cycle generates oxidizing potential to activate the pentose phosphate shunt and thus can increase nucleotide synthesis by both salvage and *de novo* pathways [56]. We speculate that the increases in nucleotides generated by p53-upregulated proline oxidase could enhance the process of DNA repair initiated by cyclin A1.

The cell cycle and apoptosis are intimately linked and a coordination and balance between these two processes are crucial for normal cell physiology [57]. The induction of a G2/M cyclin and a mitochondrial redox enzyme might allow p53 more versatility in mediating cell type-specific G2 arrest and apoptotic processes in response to certain physiological conditions of stress.

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