

# Constitutive Activation of Cyclin B1-associated cdc2 Kinase Overrides p53-mediated G<sub>2</sub>-M Arrest<sup>1</sup>

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## Abstract

Recent studies have suggested that p53 regulates the G<sub>2</sub> checkpoint in the cell cycle and that this function is required for the maintenance of genomic integrity. In this study, we investigated a regulatory role of p53 specifically in G<sub>2</sub>-M transition. Human bladder carcinoma cells lacking functional p53 were synchronized at G<sub>1</sub>-S, which is preceded by p53-mediated G<sub>1</sub> arrest. p53 expression in the synchronized cells was induced by infection with a recombinant adenovirus that encodes p53. After release from the G<sub>1</sub>-S arrest, the cells progressed to S-phase and G<sub>2</sub> but failed to enter mitosis. Biochemical analysis showed that p53 inhibits cell cycle-dependent expression of cdc2 and cyclin B1 and consequently inhibits cdc2 kinase. The role of cyclin B1-associated cdc2 kinase in p53-mediated G<sub>2</sub>-M arrest was further investigated by expression of both cyclin B1 and cdc2AF, in which inhibitory phosphorylation sites were substituted. The cells expressing both cdc2AF and cyclin B1 showed a constitutive activation of cdc2 kinase during cell cycle progression and passed through G<sub>2</sub>-M regardless of p53 expression. Therefore, inactivation of cdc2 kinase through cdc2 and cyclin B1 repression is an essential step in p53-mediated G<sub>2</sub>-M arrest.

## Introduction

After DNA damage, mammalian cells arrest at the transition from G<sub>1</sub> to S-phase (G<sub>1</sub>-S, G<sub>1</sub> checkpoint) or from G<sub>2</sub> to M (G<sub>2</sub>-M, G<sub>2</sub> checkpoint) in the cell cycle (1). Cell cycle arrest at these checkpoints prevents DNA replication and mitosis in the presence of DNA damage. Arrest at G<sub>1</sub> checkpoint results, at least in part, from p53-mediated synthesis of the cell cycle inhibitor p21, which binds to and inhibits the cdk-cyclin complexes required for the G<sub>1</sub>-S transition (2, 3). Because p53 knockout or mutant p53-expressing cells seem to arrest at G<sub>2</sub>-M following DNA damage (4), it was initially thought that p53 plays no role at G<sub>2</sub>-M transition. However, recent studies have suggested that p53 regulates the G<sub>2</sub>-M transition. We and other groups have reported that expression of wild-type p53 in human cells, using tetracycline-repressible promoter or expression of the temperature-sensitive mutant form of p53, p53val<sup>135</sup>, at permissive temperatures leads to an increase in G<sub>2</sub>-M as well as G<sub>1</sub> populations of the growth-arrested cells (5–7). However, p53 expression in nonsynchronized culture does not lead to a full clarification of the regulatory role of p53 specifically in G<sub>2</sub>-M transition. In this study, an experimental protocol was designed to express p53 in cells synchronized at G<sub>1</sub>-S, which is preceded by p53-mediated G<sub>1</sub> arrest. Thus, the p53-expressing cells progress synchronously along the cell cycle after release from G<sub>1</sub>-S arrest. This experimental system enabled us to elucidate the regulatory roles of p53 and its downstream regulators, such as cdc2

kinase in G<sub>2</sub>-M transition. In G<sub>2</sub>-M transition, the cyclin-dependent protein kinase complex, cdc2-cyclin B1 complex, plays a critical role (8). The kinase activity of cdc2 is controlled during the cell cycle both by its association with cyclin B1 and by phosphorylation and dephosphorylation on the inhibitory phosphorylation sites, Thr-14 and Tyr-15 (9, 10). The phosphorylation is catalyzed by wee1, myt1, and related kinases, whereas the dephosphorylation is facilitated by the protein phosphatase cdc25C (11). In addition, transcription of cdc2 and cyclin B1 is regulated in a cell cycle-dependent manner, beginning at G<sub>1</sub>-S and reaching maximum levels at G<sub>2</sub>-M (12, 13). Here, we provide evidences that p53 specifically blocks G<sub>2</sub>-M transition of the cell cycle through inhibition of cell cycle-dependent activation of cdc2 kinase. This inactivation of cdc2 kinase results, at least in part, from repression of cell cycle-dependent expression of cdc2 and cyclin B1. The critical role of p53-mediated inactivation of cdc2 kinase is confirmed by the finding that constitutive activation of cdc2 kinase by enforced expression of both cdc2 and cyclin B1 leads to an override of p53-mediated G<sub>2</sub>-M arrest.

## Materials and Methods

**Cell Culture and Cell Cycle Analysis.** The human bladder cancer cells, EJ, were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and were synchronized at G<sub>1</sub>-S with a modified double-thymidine block protocol (14). After synchronization, cells were infected with an adenovirus that encodes p53 (15) or the tTA virus plus the cyclin B1 or cdc2AF viruses, each at a multiplicity of infection of 40–50 plaque-forming units per cell for 3 h. After 8 h, cells were washed twice in Dulbecco's PBS, which released them from the G<sub>1</sub>-S block. Recombinant adenoviruses that encode cyclin B1, cdc2AF, and tTA were obtained from Dr. Morgan (University of California, San Francisco, CA; Ref. 14).

To analyze DNA content by flow cytometry, we trypsinized cells; fixed them in 70% ethanol; resuspended them in Dulbecco's PBS containing RNase A (50 µg/ml), 0.1% sodium citrate, and propidium iodide (50 µg/ml); and then analyzed the samples with a FACScan (Becton Dickinson, Mountain View, CA) and Lysis software. To visualize condensed chromatin, we stained ethanol-fixed cells with propidium iodide, placed them on glass slides, and examined the slides under the fluorescence microscope (Carl Zeiss, Germany).

**Protein Analysis.** Cell lysates were prepared as reported previously (16). Briefly, cells were lysed with RIPA buffer [50 mM Tris (pH 7.5), 5 mM NaCl, 1 µM EGTA, 1% Triton X-100, 50 µM NaF, 10 µM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT], and the lysates were clarified by centrifugation (1200 rpm for 10 min at 4°C). To measure histone H1 kinase activity, we incubated cell lysate (150 µg) for 12 h at 4°C with anti-cdc2 antibody (sc-34; Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-agarose (Boehringer Mannheim Inc., Indianapolis, IN). The immunoprecipitates were washed three times with RIPA buffer and twice with kinase buffer [50 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 2 mM DTT]. The immunoprecipitates in the kinase buffer were mixed with 25 µl of kinase buffer containing 20 µM ATP, 5 µCi of [<sup>32</sup>P]ATP (3000 mCi/mmol; Amersham Corp., Buckinghamshire, United Kingdom), and 1.5 µg of histone H1 (Boehringer Mannheim). After 30 min at 30°C, the reactions were terminated with SDS sample buffer, and the reaction products were analyzed by SDS-PAGE and autoradiography.

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Rabbit antibody against cyclin B1 (sc-752; Santa Cruz Biotechnology), mouse antibodies against cdc2 (sc-54; Santa Cruz Biotechnology) or p53 (sc-126; Santa Cruz Biotechnology), and goat antibody against actin (sc-1616; Santa Cruz Biotechnology) were used for immunoblot assay. Immunoblotting was performed with ECL Western blotting detection reagents (Amersham).

**Northern Blot Analysis.** Total cellular RNA was isolated with the use of Trizol solution (Life Technologies). RNA (15  $\mu$ g) was analyzed by electrophoresis in 1% agarose-formaldehyde gels and transferred to nitrocellulose filters. Hybridizations were performed with the following <sup>32</sup>P-labeled DNA probes: (a) the 1.2-kb *EcoRI/BamHI* cdc2 insert fragment containing the complete open reading frame of cdc2; (b) the 1.1-kb *KpnI/XbaI* cyclin B1 insert in pRC/CMV plasmid; (c) the 375-base fragment specific for the 3' untranslated region of  $\sigma$  was generated by PCR using EST W79136 as template and the primers 5'-ACAGGGAACTTTATTGAGAGG-3' and 5'-AAGGGCTCCGTGGAGAGG-G-3' (17). Hybridization conditions and washing procedures were optimized according to the manufacturer's instructions (ExpressHyb; Clontech Inc., Palo Alto, CA).

## Results

**p53 Inhibits G<sub>2</sub>-M Transition in the Cell Cycle.** In this study, we first established the effects of p53 expression specifically in G<sub>2</sub>-M transition of the cell cycle. The human bladder carcinoma cells, EJ, were synchronized at G<sub>1</sub>-S, which is preceded by p53-mediated G<sub>1</sub> arrest with a double-thymidine treatment. The cells were infected with recombinant adenovirus and then released from the G<sub>1</sub>-S arrest. This experimental protocol enabled us to induce p53 expression specifically in cells that had already progressed beyond the G<sub>1</sub> arrest point and to analyze the effects of p53 specifically in G<sub>2</sub>-M transition. In cells infected with a control virus lacking the *E1* gene (Ad- $\Delta$ E1; Ref. 15), mitosis occurred 8–12 h after release from the G<sub>1</sub>-S block, as indicated by flow cytometric analysis (Fig. 1A) and analysis of chromosome condensation from cells stained with propidium iodide (Fig. 1B). Uninfected cells exhibited essentially the same behavior (data not shown). In contrast, a majority of cells infected with the adenovirus that encodes p53 (Ad-p53) moved to the S and G<sub>2</sub> phases after release, but did not undergo mitosis (Fig. 1, A and B). A minor population did

not progress to S-phase, remaining at G<sub>1</sub> after release from the arrest (Fig. 1A). This G<sub>1</sub> population may be due to p53 expression before synchronization at G<sub>1</sub>-S. These results suggest that p53 inhibits G<sub>2</sub>-M transition independently of G<sub>1</sub>-S transition.

**p53 Inhibits Cell Cycle-dependent Activation of cdc2 Kinase and Expression of cdc2 and cyclin B1.** Biochemical analysis of lysates of control cells confirmed that the cyclin B1 protein level and the associated cdc2 kinase activity peaked at 8 h after release (Fig. 1C). On the other hand, the concentration of the cdc2 protein did not change significantly during cell cycle progression from G<sub>1</sub>-S to G<sub>2</sub>-M (Fig. 1C). However, in the cells infected with Ad-p53, cell cycle-dependent expression of cyclin B1 and cdc2 kinase activity was completely inhibited (Fig. 1C). Furthermore, the cdc2 protein level decreased during cell cycle progression, becoming barely detectable by 24 h (Fig. 1C).

Transcriptions of the *cdc2* and *cyclin B1* genes have been shown to be regulated in a cell cycle-dependent manner, beginning at G<sub>1</sub>-S and reaching maximum levels at G<sub>2</sub>-M (12, 13). In cells infected with a control virus, cdc2 and cyclin B1 mRNA peaked at 8–10 h (Fig. 2A). However, in cells infected with Ad-p53, cell cycle-dependent increases in cdc2 and cyclin B1 mRNA levels were abolished (Fig. 2A).

Recently, it was shown that transcription of the *14-3-3 $\sigma$*  gene, which is a new member of the *14-3-3* gene family, is induced in a p53-dependent manner after DNA damage and that its overexpression leads to arrest at G<sub>2</sub>-M (17). We examined *14-3-3 $\sigma$*  transcription after release from the G<sub>1</sub>-S arrest (Fig. 2B). Transcription of *14-3-3 $\sigma$*  remained at the basal level during cell cycle progression in cells infected with a control virus but was induced in cells infected with a virus that encodes p53 (Fig. 2B).

**Activation of cdc2 Kinase Overrides p53-mediated G<sub>2</sub>-M Arrest.** To verify the role of the cdc2-cyclin B1 complex in p53-mediated G<sub>2</sub>-M arrest, we examined whether constitutive activation of cyclin B1-associated cdc2 kinase overrides p53-mediated cell cycle arrest at the G<sub>2</sub> checkpoint. To achieve constitutive activation of cdc2 kinase, we used recombinant adenoviruses that encode cyclin B1 or

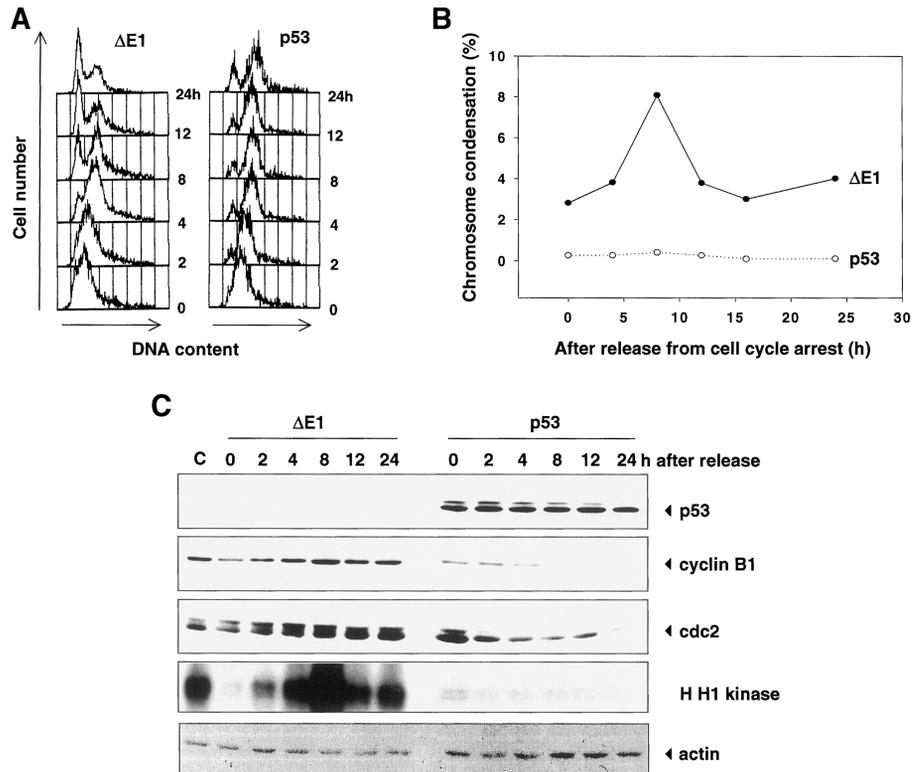
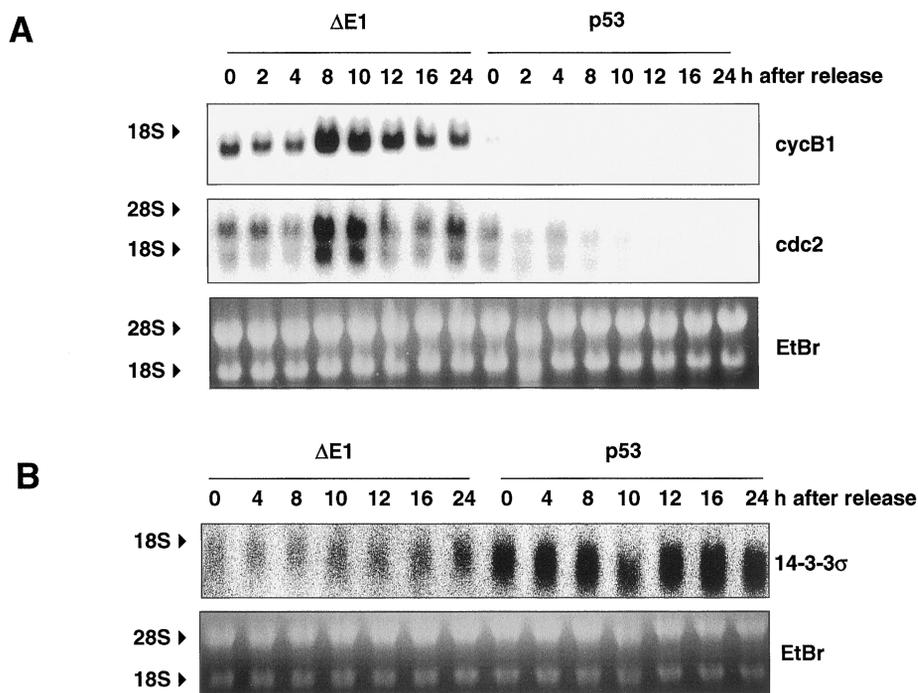


Fig. 1. Effects of p53 on synchronous progression of cell cycle. A, EJ cells were synchronized at G<sub>1</sub>-S boundary by a double-thymidine treatment and infected for 3 h with a recombinant adenovirus that encodes p53 (Ad-p53; p53) or a control virus (Ad- $\Delta$ E1;  $\Delta$ E1). After release from the growth arrest, cells were harvested at the indicated time points, fixed in 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry for DNA content. B, cells from the same experiment as in A were fixed in ethanol:acetic acid (3:1, v/v) at the indicated time points and examined by microscopy for the presence of condensed chromosomes. The fractions of Ad- $\Delta$ E1 (●) or Ad-p53 (○) cells containing condensed chromosomes were determined by counting at least 600 cells for each sample. C, cell lysates were prepared at the indicated time points and normal growing stage (C). Equal amounts of cell lysates (15  $\mu$ g) were subjected to Western blot analysis with antibodies against the indicated proteins. Expression of actin protein was examined as a loading control. Histone H1 kinase (*H H1 kinase*) activity was measured in immunoprecipitates with anti-cdc2.  $\Delta$ E1, Ad- $\Delta$ E1; p53, Ad-p53

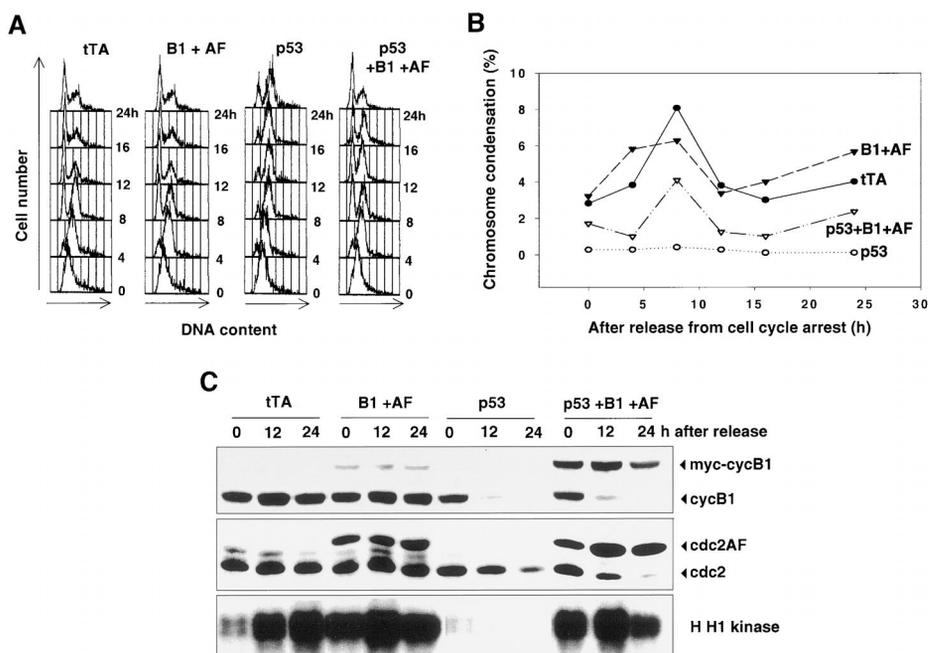
Fig. 2. Effects of p53 on cell cycle-dependent transcription of *cdc2* and cyclin B1. *A* and *B*, EJ cells were arrested at G<sub>1</sub>-S boundary and infected for 3 h with a control virus (Ad- $\Delta$ E1;  $\Delta$ E1) or p53. Cells were released from the thymidine block and harvested at the indicated time points. Total RNA (15  $\mu$ g) was separated on a 1% agarose-formaldehyde gel and transferred to nylon membranes. The filters were hybridized with *cdc2* or cyclin B1 (*A*), or 14-3-3 $\sigma$  probe (*B*). Ethidium bromide (*EtBr*) staining is presented as loading control.



the dominant mutant form of *cdc2*, *cdc2AF*, in which Thr-14 and Tyr-15 are changed to alanine and phenylalanine, respectively (14). The *cyclin B1* and *cdc2AF* genes are under the control of a tetracycline-repressible promoter; expression from this promoter requires that cells are co-infected with a recombinant adenovirus that encodes the tTA transactivator (18). These synchronized cells were infected with adenoviruses that encode tTA and both cyclin B1 and *cdc2AF*. Expression of both *cdc2AF* and cyclin B1 resulted in a constitutive activation of the *cdc2* kinase regardless of positions in the cell cycle after release (Fig. 3C), whereas expression of either *cdc2AF* or cyclin B1 did not significantly increase the *cdc2* kinase activity (data not shown). Whereas cells expressing p53 had no detectable *cdc2* kinase activity, cells expressing p53 together with *cdc2AF* and cyclin B1

showed a constitutive activation of *cdc2* kinase regardless of p53 expression (Fig. 3C). Cells expressing tTA alone or tTA plus both *cdc2* and cyclin B1 behaved identically to control cells infected with Ad- $\Delta$ E1 in cell cycle progression and timing on mitosis entry (Fig. 3, *A* and *B*). Whereas cells expressing p53 were arrested at G<sub>2</sub>-M for at least 24 h after release, cells expressing p53 and both *cdc2AF* and cyclin B1 did not arrest at G<sub>2</sub>-M, entering the next G<sub>1</sub> at 8–12 h, and chromosome condensation occurred normally, as with control cells (Fig. 3, *A* and *B*). However, the cells were arrested at the next G<sub>1</sub>, which is another arrest point mediated by p53 expression (Fig. 3A). These results suggest that constitutive activation of *cdc2* kinase permits cell to progress through G<sub>2</sub>-M arrest, but not G<sub>1</sub> arrest, in the presence of p53.

Fig. 3. Effects of constitutive activation of *cdc2*-cyclin B1 in p53-mediated G<sub>2</sub>-M arrest. *A*, EJ cells were arrested at G<sub>1</sub>-S boundary by a double-thymidine treatment, infected for 4 h with recombinant adenoviruses that encode tTA (*tTA*), p53 (*p53*), multiple viruses that encode tTA plus cyclin B1 and *cdc2AF* (*B1 + AF*), or both cyclin B1 and *cdc2AF*, and Ad-p53 (*p53 + B1 + AF*). As in the legend to Fig. 1, cells were released from the thymidine block, harvested at the indicated time points, fixed in 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. *B*, fractions of cells containing condensed chromosomes were determined by counting >600 cells for each sample. ●, tTA; ○, p53; ▼, B1 + AF; ▽, p53 + B1 + AF. *C*, cell lysates prepared at the indicated time points were subjected to immunoblotting with antibodies against cyclin B1 or *cdc2*. Histone H1 kinase (*H1 kinase*) activity was measured in immunoprecipitates with anti-*cdc2*.



## Discussion

In this study, we found that p53 did not affect synchronous progression of the cell cycle from G<sub>1</sub>-S to G<sub>2</sub> but that it did block entry to mitosis (Fig. 1). Cell cycle-dependent transcriptions of *cdc2* and *cyclin B1* were inhibited in the presence of p53 (Fig. 2A). This transcriptional inhibition results in failure in cell cycle-dependent activation of cdc2 kinase, which peaks at G<sub>2</sub>-M transition in control cells (Fig. 1C). Activation of cdc2 kinase is an essential step for mitosis entry: inactivation of cdc2 kinase by expression of a dominant negative mutant form of cdc2 results in cell cycle arrest at G<sub>2</sub>-M (19). These results suggest that p53 inhibits mitosis entry by inactivating cdc2 kinase, which, at least in part, results from repression of *cdc2* and *cyclin B1* transcription.

In the human colorectal cancer cell line, HCT116, expression of the *14-3-3σ* gene is induced in a p53-dependent manner after DNA damage, and its overexpression leads to arrest at G<sub>2</sub>-M (17). The 14-3-3σ protein has been proposed to induce G<sub>2</sub>-M arrest by binding to the nonfunctional phosphorylated form of cdc25C phosphatase, which in turn prevents activation of cdc2 (20). Transcription of *14-3-3σ* was induced in EJ cells infected with Ad-p53 during cell cycle progression (Fig. 2B). If p53 inhibits cdc2 kinase through accumulation of 14-3-3σ, the phosphorylated fractions of cdc2 should increase in cells infected with an adenovirus that encodes p53. However, as shown in Fig. 1C, phosphorylated forms of cdc2 were decreased and finally became undetectable after release from G<sub>1</sub>-S arrest in the presence of p53. These results suggest that 14-3-3σ does not affect cdc2 phosphorylation in EJ cells infected with Ad-p53. Recently, Winters *et al.* (21) reported that inactivation of cdc2 kinase after DNA damage results from nuclear translocation rather than inhibitory phosphorylation of cdc2 in p53-expressing H1299 cells. Therefore, it is unlikely that p53 inhibits cdc2 kinase through the inhibitory phosphorylation of cdc2. Instead, p53 inhibits cdc2 kinase activity through repression of cyclin B1 and cdc2. We previously reported that p53 represses cdc2 and cyclin B1 promoters (16); therefore, it is likely that p53-mediated repression of cdc2 and cyclin B1 mRNA levels results from inactivation of the promoters. The NF-Y transcription factor, which binds to the CCAAT sequences on these promoters, seems to mediate repression by p53 (16). Recently, it was suggested that NF-Y is involved in cell cycle-dependent activation of cyclin B1 promoter (22). Taking observations of this and other groups together, we propose that p53 inactivates the G<sub>2</sub>-specific cyclin-cdk complex, cyclin B1-cdc2, through at least three different routes: transcription repression of *cdc2* and *cyclin B1* (Fig. 2A and Ref. 16); transcription activation of *14-3-3σ* (17); and nuclear localization of cyclin B1-cdc2 (21).

Recently, it was reported that transient overexpression of cyclin B1 alone leads to decrease in G<sub>2</sub>-M population in growth-arrested cells by a temperature-sensitive *p53* gene (23). However, expression of either cdc2AF or cyclin B1 at normal levels, using a tetracycline-repressible promoter in the HeLa S3 cell line, has only minor effects on cell cycle progression at G<sub>2</sub>-M (14). Although expression of cyclin B1 alone did not affect to the G<sub>2</sub>-M arrest that follows DNA damage, expression of both cdc2 and cyclin B1 reduced the damage-induced G<sub>2</sub> delay (14). In this study, we expressed cyclin B1 and/or cdc2AF by use of a tetracycline-repressible promoter in the synchronized p53-deficient EJ cells. Expression of either cdc2AF or cyclin B1 had no significant effects in the p53-mediated G<sub>2</sub>-M arrest (data not shown). By contrast, when both cdc2AF and cyclin B1 were expressed, cdc2 kinase was activated regardless of cell cycle position. Under this condition, cells pass through G<sub>2</sub>-M in the presence of p53. These results suggest that inactivation of cyclin B1-cdc2 is an essential step of p53-mediated

G<sub>2</sub>-M arrest. Previous reports have indicated that cyclin B1 destruction is required for exit from mitosis (14, 24). However, the cells expressing cdc2AF and cyclin B1 normally exited from mitosis and entered the next G<sub>1</sub> phase (Fig. 3), presumably because of the relatively low expression of cyclin B1 in those cells.

In summary, our study clearly demonstrates that inhibition of cdc2 kinase through p53-mediated cdc2 and cyclin B1 repression is essential for G<sub>2</sub>-M arrest.

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