

p53 and DNA-dependent protein kinase catalytic subunit independently function in regulating actin damage-induced tetraploid G1 arrest

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Abbreviations: ATM, ataxia telangiectasia mutated protein; DCB, dihydrocytochalasin B; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; PTX-2, pectenotoxin-2

Abstract

We previously reported that the p53 tumor suppressor protein plays an essential role in the induction of tetraploid G1 arrest in response to perturbation of the actin cytoskeleton, termed actin damage. In this study, we investigated the role of p53, ataxia telangiectasia mutated protein (ATM), and catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) in tetraploid G1 arrest induced by actin damage. Treatment with actin-damaging agents including pectenotoxin-2 (PTX-2) increases phosphorylation of Ser-15 and Ser-37 residues of p53, but not Ser-20 residue. Knockdown of ATM and DNA-PKcs do not affect p53 phosphorylation induced by actin damage. However, while ATM knockdown does not affect tetraploid G1 arrest, knockdown of DNA-PKcs not only perturbs tetraploid G1 arrest, but also results in formation of polyploidy and induction of apoptosis. These results indicate that DNA-PKcs is essential for the maintenance of actin damage induced-tetraploid G1 arrest in a p53-independent manner. Furthermore, actin damage-induced p53 expression is not observed in cells synchronized at G1/S of the cell cycle, implying that p53 induction is due to actin damage-induced tetraploidy rather than pertur-

bation of actin cytoskeleton. Therefore, these results suggest that p53 and DNA-PKcs independently function for tetraploid G1 arrest and preventing polyploidy formation.

Keywords: actin cytoskeleton; ataxia telangiectasia mutated protein; DNA-activated protein kinase; pectenotoxin 2; tumor suppressor protein p53

Introduction

Various cell cycle checkpoints ensure coordinated progression of the cell cycle (Hartwell and Weinert, 1989) and checkpoint defects result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumorigenesis (Paulovich *et al.*, 1997). A molecular understanding of various cellular checkpoints should therefore provide a basis for the development of new therapeutic approaches for many cancers with abnormalities in checkpoint functions (Stewart and Pietsenpol, 1999). The G1 checkpoint permits repair prior to replication, whereas the G2 checkpoint allows repair of the genome prior to its mitotic segregation. The p53 tumor suppressor gene, which is suppressed by mutation in approximately one-half of human tumors (Hollstein *et al.*, 1991), has been shown to be integral to both the G1 (Kuerbitz *et al.*, 1992) and G2 (Bunz *et al.*, 1998) DNA damage checkpoint machinery. In addition to DNA damage, spindle damage induces a transient arrest at the metaphase-anaphase transition point (Rudner and Murray, 1996; Amon, 1999), but eventually escape from this block and exit mitosis without proper segregation of sister chromatids and cytokinesis (Jordan *et al.*, 1991, 1996; Torres and Horwitz, 1998). Therefore, spindle-damaged cells arrest at a G1-like state with an intact nucleus containing 4N DNA, but without ever completing mitosis (Minn *et al.*, 1996; Lanni and Jacks, 1998). This is termed a tetraploid G1 state.

Actin depolymerizing agents such as pectenotoxin-2 (PTX-2) and dihydrocytochalasin B (DCB), which inhibit cytokinesis by depolymerizing actin filaments,

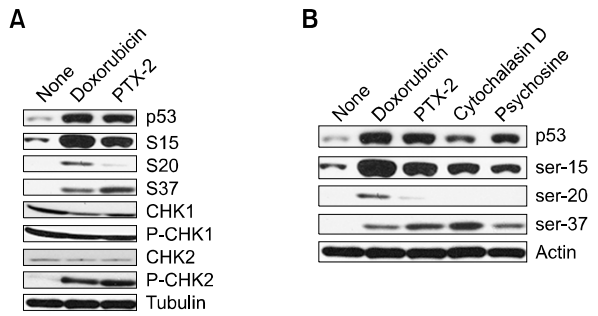


Figure 1. Phosphorylation of p53 upon actin damage. (A) HCT116 colon carcinoma cells were treated with PTX-2 (100 ng/ml) for 72 h or doxorubicin (150 nM) for 24 h. (B) HCT116 cells were treated with actin-damaging agents, such as PTX-2 (100 ng/ml), cytochalasin D (10 μM), and psychosine (50 μM) for 72 h. Cell extracts were subjected to Western blot analysis with the indicated antibodies.

also lead to tetraploid G1 arrest (Andreassen *et al.*, 2001; Chae *et al.*, 2005). Since actin-damaging agents do not affect spindle function and chromatid segregation, but only inhibit cytokinesis, the tetraploid G1 checkpoint seems to be a general checkpoint control acting in G1 to recognize tetraploid cells and induce their arrest and thereby prevent the generation of aneuploidy (Margolis *et al.*, 2003; Margolis, 2005).

We previously reported that loss of p53 sensitizes tumor cells to actin damage induced by treatment with actin-depolymerizing or knotting agents (Chae *et al.*, 2005). Upon actin damage, Bim expression was induced in tumor cells lacking functional p53 followed by conformational changes of Bax protein (Chae *et al.*, 2005). Furthermore, induction of Bim-mediated apoptosis by actin damage in p53-deficient cells results from constitutive cdk2 activation and its associated genomic instability (Chae *et al.*, 2008). In this study, we demonstrate that actin damage, like DNA damage, induces phosphorylation of Ser-15 and Ser-37 residues of p53 protein, but the upstream signaling pathways leading to the phosphorylation was different in between DNA and actin damage responses. However, although DNA-PKcs is not responsible for phosphorylation of p53 protein, it was shown to be essential for the maintenance of tetraploid G1 arrest following actin damage and cytokinesis failure.

Results and Discussion

We previously reported that actin damage induces tetraploid G1-arrest by activating the p53 tumor suppressor protein, which leads to inactivation of Cdk2 (Chae *et al.*, 2005, 2008). In this study, we first addressed the molecular mechanism underlying

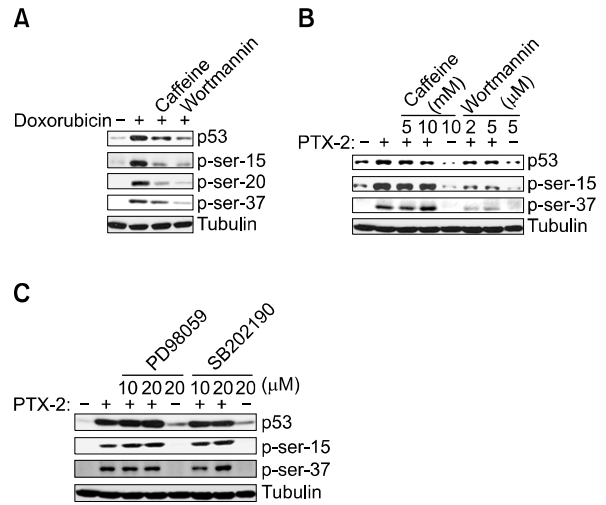


Figure 2. Inhibition of p53 phosphorylation by PI3 kinase inhibitors. (A) HCT116 cells were co-treated with a DNA damaging agent, doxorubicin (200 nM), and either caffeine or wortmannin for 24 h. (B) Cells were co-treated with PTX-2 (100 ng/ml) and the indicated dose of caffeine or wortmannin for 72 h. (C) Cells were co-treated with PTX-2 (100 ng/ml) and the indicated dose of PD98059 or SB202190 for 72 h. Cell extracts were subjected to Western blot analysis with the indicated antibodies.

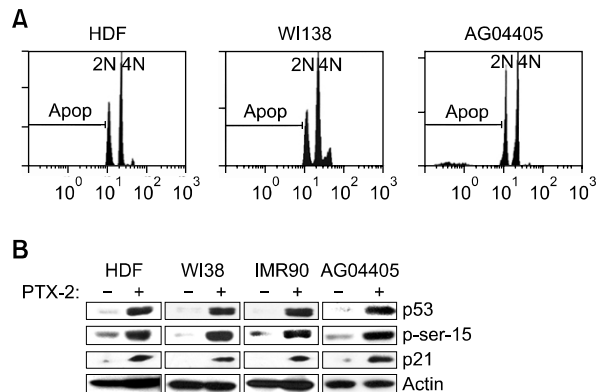


Figure 3. p53 phosphorylation in ATM-knockout cells. (A) Flow cytometric analysis of human normal fibroblasts HDF and WI38, and ATM-knockout fibroblasts AG04405 after treatment with PTX-2 (100 ng/ml) for 72 h. (B) Cell extracts were prepared from cells treated as in (A) and subjected to Western blot analysis with the indicated antibodies.

p53 induction by actin damage. Since it has been clearly shown that p53 accumulation is largely dependent on the phosphorylation state of the amino-terminal region of p53 protein (Giaccia and Kastan, 1998; Ashcroft *et al.*, 1999; Oren, 1999), we examined major phosphorylation sites of the N-terminal region of p53. While a DNA damaging agent, doxorubicin, induced phosphorylation of serine residues located at the amino terminal, namely Ser-15, -20, and -37, all of the actin inhibitors tested here induced phosphorylation of Ser-15 and -37, but left Ser-20 unphosphorylated (Figures 1A and

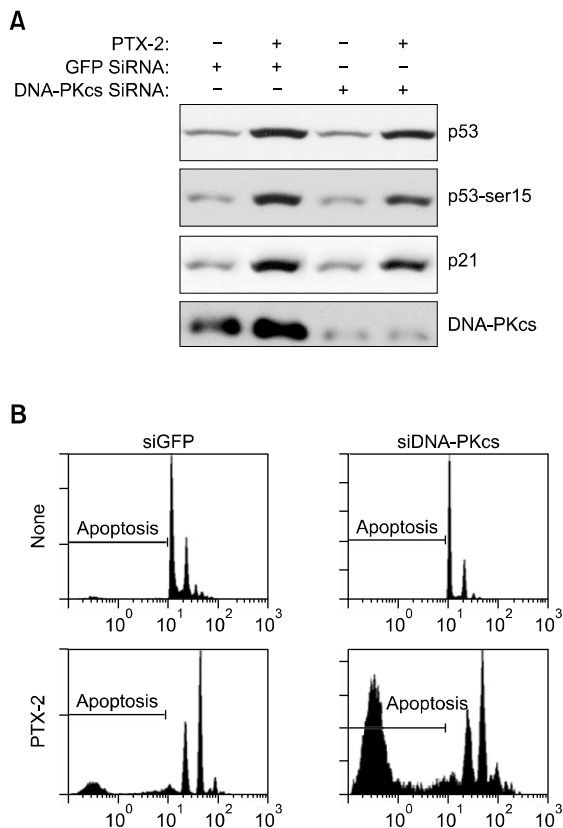


Figure 4. p53 phosphorylation and apoptotic death in DNA-PKcs knock-down cells. (A) HCT116 cells were transfected with siRNA targeting DNA-PKcs or GFP as a control. Cells were treated with PTX-2 2 days after siRNA transfection. Cell extracts were subjected to Western blot analysis with the indicated antibodies. (B) Flow cytometric analysis of HCT116 cells transfected with GFP siRNA or DNA-PKcs siRNA after treatment with PTX-2 (100 ng/ml) for 72 h.

1B).

It was further examined the effects of caffeine and wortmannin, two PI3 kinase inhibitors known to inhibit p53 phosphorylation upon DNA damage (Ui *et al.*, 1995; Kaufmann *et al.*, 2003), on the phosphorylation of Ser-15 and -37 of p53. Both inhibitors blocked DNA damage-induced phosphorylation of all three serine residues in p53 (Figure 2A). However, we found that the PI3 kinase inhibitors function differentially in the phosphorylation of p53 induced by actin damage. While caffeine did not inhibit PTX-2-induced phosphorylation of Ser-15 and Ser-37, wortmannin blocked their phosphorylation (Figure 2B). PD98059 and SB212090, which inhibit ERK1 and p38, respectively, did not affect phosphorylation of Ser-15 and Ser-37 (Figure 2C). Thus, these results suggest that a caffeine-insensitive, but a wortmannin-sensitive kinase is responsible for actin damage-induced phosphorylation of the Ser-15 residue of p53.

Since both Ser-15 and Ser-37 of p53 are

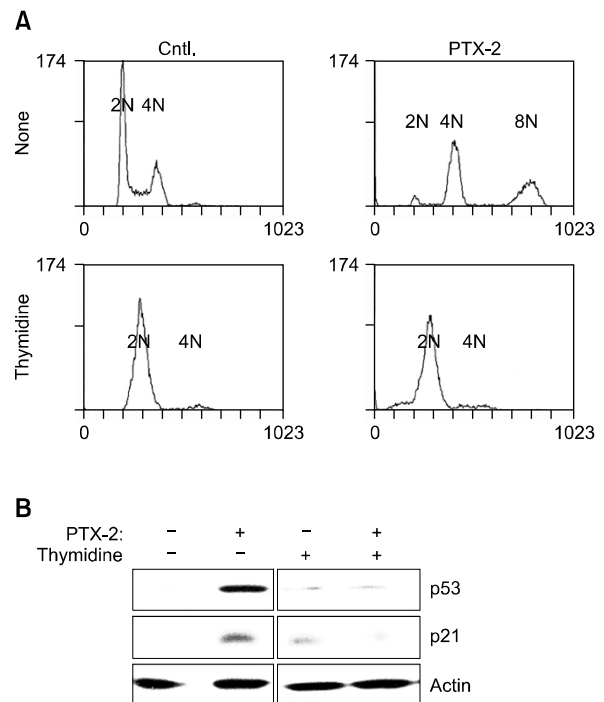


Figure 5. PTX-2 does not induce p53 expression in G1/S-arrested cells. (A) HCT116 cells were treated with PTX-2 (100 ng/ml) for 72 h under conditions of normal growth or G1-synchronization by treatment with thymidine as described in Methods. DNA content was analyzed by flow cytometry. (B) Western blot analysis of HCT116 cells treated as in (A).

phosphorylated by ATM kinase in response to DNA damage, ATM-positive and -deficient cells were examined for PTX-2 dependent phosphorylation of those residues of p53. AG04405 cells, which are derived from an AT patient deficient in ATM, and normal fibroblasts such as HDF, WI38, and IMR-90, exhibited cell cycle arrest and phosphorylation of p53 protein upon actin damage (Figures 3A and 3B), indicating that actin damage induces phosphorylation of p53 Ser-15 in an ATM-independent manner. Therefore these results suggest that upstream signaling pathway leading p53 phosphorylation is different in between DNA and actin damage responses.

Among PI3 kinases, DNA-PKcs is the most resistant to inhibition by caffeine (Sarkaria *et al.*, 1999). Therefore, we further examined the role of DNA-PKcs in p53 phosphorylation. Induction of p53 expression and Ser-15 phosphorylation was not affected by the knockdown of DNA-PKcs (Figure 4A). However, unexpectedly, DNA-PKcs knockdown cells exhibited enhanced apoptotic death following treatment with PTX-2 in association with an increased number of polyploid cells (Figure 4B). Therefore, these results suggest that DNA-PKcs does not account for p53 phosphorylation, but is

involved in the maintenance of tetraploid G1 arrest following actin damage.

Because of p53-independent function of DNA-PKcs in tetraploid G1 arrest, we further examined whether p53 induction is a result of actin damage or polyploidy. To end this, cells were first arrested at early S phase by double thymidine block (Park *et al.*, 2000). While cells treated with PTX-2 alone exhibited tetraploid (4N) and polyploidy (8N), those synchronized by double thymidine block kept arresting at G1/S after the PTX-2 treatment (Figure 5A). However, p53 induction was not observed in cells synchronized by the thymidine block upon treatment with PTX-2 (Figure 5B), implying that the perturbation of actin cytoskeleton by PTX-2 does not induce p53 in cells arrested at G1/S phase. Therefore, these results suggest that p53 induction upon actin damage is not merely a result of perturbation of the actin cytoskeleton. Instead, cell may recognize actin damage-induced polyploidy as a stress signal inducing p53. Taken together these, p53 and DNA-PKcs function independently to sustain tetraploid G1 arrest and prevent polyploidy formation.

Methods

Cell culture

Human colorectal cancer cell line HCT116, ATM-deficient fibroblast cell line AG04405, and human normal fibroblast cell lines IMR90 and WI38 were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (Life Technologies, Inc., USA) and penicillin-streptomycin (50 U/ml). For knockdown of DNA-PKcs expression, HCT116 cells were transfected with siRNA targeting DNA-PKcs, 5'-AAAGGGCCAAGCTGTCACTCT-3' (Dharmacon Research) using Lipofectamine 2000 (Invitrogen).

Cell cycle synchronization at G1/S of the cell cycle

HCT116 cells were synchronized at G1/S with a modified double thymidine block protocol as previously described (Park *et al.*, 2000). Cells were plated at 0.3×10^6 cells /100-mm dish and 0.1×10^6 cells / 60-mm dish. After 1 day, cells were arrested by treatment with thymidine (5 mM, Sigma Corp.) for 17 h, and were then released from the arrest by washing in Dulbecco's phosphate buffered saline (8 mg/ml NaCl, 0.2 mg/ml KCl, 0.1 mg/ml CaCl_2 , 0.1 mg/ml MgCl_2 , 2.31 mg/ml $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 mg/ml KH_2PO_4) and replenishing with thymidine-free medium. After 9 h, cells were subjected to a second thymidine treatment.

Cell cycle analysis

For DNA content analysis, 1×10^6 cells were harvested by trypsinization and fixed by rapid submersion in 1 ml cold

70% ethanol. After fixation at -20°C for at least 1 h, cells were pelleted and resuspended in 1 ml staining solution (50 $\mu\text{g/ml}$ propidium iodide, 50 $\mu\text{g/ml}$ RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8), and analyzed with FACScan (Becton Dickinson) using Lysys software (Chae *et al.*, 2004; Jung *et al.*, 2004).

Western blot analyses

Twenty micrograms of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PolyScreen membranes (NEN). The membranes were subsequently blocked with 5% nonfat dry milk in Tris-buffered saline Tween20 buffer (Jung *et al.*, 2001) and probed with antibodies. Primary antibodies were detected with a horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat secondary antibodies with enhanced chemiluminescence detection (Amersham, UK). Primary antibodies used were followings: anti-p53, anti-p-ser15, anti-p-ser20, anti-p-ser37, anti-actin, anti-p21, anti-DNA-PKcs, (Santa Cruz Biotechnology, CA); and α -tubulin (Sigma-Aldrich, MO).

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