Cdk5 Activator-binding Protein C53 Regulates Apoptosis Induced by Genotoxic Stress via Modulating the G2/M DNA Damage Checkpoint* [S]

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Mammalian cells have developed sophisticated mechanisms to cope with DNA damage induced by either normal metabolic processes or genotoxic stresses. In response to genotoxic stress, cells may either undergo cell cycle arrest and DNA repair or commit suicide if the damage is beyond repair. Among many factors that influence cellular decision-making under genotoxic stress, DNA damage checkpoints play crucial roles in regulation of cell cycle arrest and cell death. DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage (1). Based upon the interphase transition that is inhibited by DNA damage, three major checkpoints are defined as the G1/S, intra-S, and G2/M checkpoints (2). In response to DNA damage, these checkpoints inhibit cell cycle progression from G1 to S (the G1/S checkpoint), DNA replication (the intra-S checkpoint), or G2 to mitosis (the G2/M checkpoint), respectively (2). Although DNA damage checkpoints are crucial for maintaining DNA integrity in normal cells, they may also present a great challenge to cancer treatment because they may arrest cell cycle and trigger DNA repair in tumor cells, thereby reducing the effectiveness of many conventional therapies that cause DNA damage. Therefore, many efforts have been made to target specific checkpoints to sensitize tumor cells to conventional therapies (3).

Orderly progression of normal cell cycle is closely monitored and tightly controlled by multiple cell cycle checkpoints. A recent study indicates that the same biochemical pathways and relevant proteins are involved in both cell cycle control and DNA damage checkpoints. One of the major players of the G2/M checkpoint is cyclin-dependent kinase 1 (Cdk1), formerly called cdc2-cyclin B1 complex that is absolutely required for the entry of mitosis (4–7). Cdk1 activation is a multistep process that begins with the increase of cyclin B1 transcription during G2 phase. Meanwhile, Cdk1 is inhibited by phosphorylation on residues Thr-14 and Tyr-15 (by Myt1 and Wee1 kinases, respectively). At the onset of mitosis, Cdk1 is activated through dephosphorylation of Thr-14 and -15 that is carried out by dual phosphatase CDC25C as well as phosphorylation on Thr-161 by Cdk-activating kinase (a heterodimer of Cdk7 and cyclin H). Once active, Cdk1-cyclin B1 complex phosphorylates CDC25C to enhance its phosphatase activity, resulting in a positive feedback loop. Meanwhile, active Cdk1-cyclin B1 translocates from cytoplasm to nucleus during early mitosis and phosphorylates nuclear substrates, leading to nuclear envelope disassembly, chromosome condensation, and separation of sister chromatids. At the end of metaphase, cyclin B1 is destroyed by anaphase promoting complex to allow mitosis to proceed. In response to DNA damage, the G2/M checkpoint system functions to arrest cell cycle in part by inhibiting Cdk1-cyclin B1 activity. The phosphatidylinositol 3-kinase family of protein

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The abbreviations used are: Cdk, cyclin-dependent kinase; ATM, ataxia telangiectasia-mutated; ATR, A-T and rad3-related; siRNA, small interfering RNA; shRNA, small hairpin RNA; TNF, tumor necrosis factor; zVAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone; EGFP, enhanced green fluorescent protein; DN, dominant negative.
kinases including ATM (ataxia telangiectasia-mutated) and ATR (A-T and rad3-related) transduce the signal of DNA damage and phosphorylate and activate checkpoint kinases checkpoint kinase 1 and 2, which in turn phosphorylate CDC25C (3). Phosphorylated CDC25C is sequestered by 14-3-3 away from Cdk1, thereby preventing dephosphorylation and activation of Cdk1 (8, 9). Another mechanism of the G2/M checkpoint control is nuclear exclusion of cyclin B1 (10, 11). Phosphorylation of cyclin B1 is critical for subcellular localization of Cdk1-cyclin B1 complex (10, 12–15). At the onset of mitosis, phosphorylation of four serine residues (Ser-126, -128, -133, and -147) at the N-terminal region of human cyclin B1 that contains both cytoplasmic retention sequence and nuclear export sequence causes an inhibition of nuclear export and enhances nuclear import, resulting in a net accumulation of cyclin B in nucleus.

Cdk1-cyclin B1 has also been demonstrated to play a critical role in regulation of apoptosis in a number of experimental systems (16). Increased Cdk1 activity has been observed in various apoptotic conditions (17–19). Furthermore, overexpression of active Cdk1-cyclin B1 promotes mitotic catastrophe and apoptosis (10, 20), and inhibition of the Cdk1-cyclin B1 complex by dominant-negative cdk1 mutant, antisense constructs, or chemical inhibitors prevents apoptosis (18, 21, 22). Yet the molecular mechanism of Cdk1-mediated apoptosis remains largely unclear.

In this study we report here that Cdk5 activator p35-binding protein C53 plays a critical role in regulating genotoxic stress-induced apoptosis through modulating the G2/M DNA damage checkpoint. By overexpressing the G2 checkpoint-mediated inhibition of Cdk1-cyclin B1 function, ectopic expression of C53 may represent a novel approach for chemotherapeutic sensitization of cancer cells.

**MATERIALS AND METHODS**

*Tissue Culture Cells and Reagents—HeLa cells (from ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. TNFα, genotoxic reagents, and chemicals were purchased from Sigma. Purified Cdk1-cyclin B1 was purchased from Upstate.*

*In Vitro Expression Cloning—Construction of mouse spleen cDNA small pool library and *in vitro* expression cloning was described (23). cDNA Cloning, Construction of Expression Vectors, and Site-specific Mutagenesis—Human C53 cDNA was amplified using primers containing Sall and KpnI sites and EST clone BE336801 (Invitrogen) as a template. Amplified C53 cDNA was sequenced and subcloned in-frame into pcMV-5a (Sigma), and the resulting construct was used for expression of the C-terminal FLAG-tagged C53 in mammalian cells. Site-specific mutagenesis was performed using the QuikChange site-specific mutagenesis kit (Stratagene) according to the manufacturer’s protocol. Mutations were confirmed by DNA sequencing.*

*Production and Purification of C53 Antibodies—His-tagged murine C53 fusion protein was purified using a nickel column (Novagen) and injected into three rats to generate polyclonal antibodies from rat. Rat polyclonal antibody (used for immunostaining assay) was affinity-purified. Two rabbit polyclonal antibodies were generated against two peptides (residues 242–256, KRGNSTYVEWTGTE, and residues 491–506, SKRYSRGPVNLMTGSL). sera were further purified using affinity chromatography.*

*Co-immunoprecipitation, Immunoblotting, Immunostaining, and Antibodies Used in This Study—For co-immunoprecipitation assay, HeLa cells (5 × 10⁶) were harvested and resuspended in the lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 0.2% Nonidet P-40, 50 mM NaF, supplemented with protease inhibitor cocktails). After extraction, 5 μg of cyclin B1 antibody was incubated with cell lysate for 2 h at 4°C followed by the addition of 10 μl of protein A/G beads (Pierce). After three washes of lysis buffer, the bound proteins were eluted with 0.1 M glycine, pH 2.5. The samples were subjected to SDS-PAGE and immunoblotting.*

*Immunostaining of cytochrome *c*, C53, and cyclin B1 and immunoblotting was performed as described (23, 24). The following antibodies were used in this study: caspase-2 and -8 (23, 24), caspase-3 and -7, poly(ADP-ribose) polymerase, phospho-cyclin B1 (Ser-147), Cdk1 and phospho-Cdk1 (Tyr-15) (Cell Signaling), caspase-9, cyclin B1, Hsp90 (Santa Cruz Biotechnology), Bcl-2 (DAKO), Bim and Apaf-1 (Stressgen), IkK (Santa Cruz Biotechnology), cytochrome *c*, FADD (FAS-associated death domain protein), XIAP (X-chromosome-linked inhibitor of apoptosis), and CIAP1 (cellular inhibitor of apoptosis 1) (BD Biosciences), Bax and histone H1 cloneAE-4 (Upstate Biotechnology), p21 and p53 (Oncogene), and FLAG M2 and tubulin (Sigma). All of C53 antibodies were generated by the Li laboratory as described above.*

*Caspase Activity Assay—Caspase activity was analyzed by Apo-one caspase assay kit (Promega) according to the manufacturer’s instruction.*

*Small Interfering RNA (siRNA) Synthesis and Construction of Small Hairpin RNA (shRNA) Expression Vectors—C53 siRNAs were designed according to Elbashir et al. (25) and synthesized by Qiagen. siRNA-1 for human C53 is (5’-GCGAGAUUCCCAAGUGGCACG-3’), whereas siRNA-2 is (5’-GCGGACCUCUUGGCAACAGG-3’). To construct shRNA expression vectors, we ligated shRNA oligonucleotide template into linearized pSIREN-RetroQ vector (Clontech) to generate pSIREN-RetroQ-C53 ex-pression C53 shRNA. siRNA-1 and -2 were designed and synthesized according to guidelines provided by Clontech.*

*Establishment of C53-deficient Stable Clones—HeLa cells (10⁶) were transfected with pSIREN-RetroQ-C53 plasmid or pSIREN-RetroQ-Luc (expressing luciferase siRNA). After a 48-h incubation, cells were treated with puromycin (2 μg/ml) for 3–4 days. Single clones were obtained by serial dilutions, and C53 expression was examined by immunoblotting.*

*Cell Death and Cell Viability Assays—For trypan blue exclusion assay, cells were trypsinized, combined and harvested with floating cells, and subsequently incubated with 0.4% trypan blue solution (Sigma) for 5 min. Blue cells were scored as dead cells. Three independent assays were performed for each experiment, and at least more than 200 cells were scored for. For annexin V assay, cells (10⁶) were partially trypsinized, harvested, and incubated with annexin V and propidium iodide (Oncogene) in the binding buffer according to the supplier’s instruction. The samples were subjected to flow cytometry analysis.*

*Cell viability was measured by CellTiter-Blue assay (Promega) according to the supplier’s instruction. For ectopic expression of C53 in normal and C53-deficient cells, the constructs expressing C53 and its mutant were transfected into HeLa cells along with EGFP marker using Lipofectamine 2000 (Invitrogen). After a 24-h incubation, cells were treated with genotoxic reagents at a variety of agents at the indicated concentrations for either 24 or 48 h. The percentage of cell death was scored by counting GFP-positive cells with DNA staining. Small and round cells with condensed, and fragmented nuclei were scored as dead cells, whereas flat and big cells with normal nuclei were scored as live cells. Each experiment was independently repeated as a triplicate.*

*Cell Cycle Analysis—Cells were trypsinized, washed in phosphate-buffered saline and fixed with ethanol at –20°C. Cells were then washed in phosphate-buffered saline again and resuspended in phosphate-buffered saline containing RNase A (5 μg/ml) and propidium iodide (10 μg/ml). A total of 10,000 labeled nuclei were analyzed in a FACScan Flow Cytometer (BD Biosciences).*

*Cdk1 Kinase Assay—Cells were detached from dishes with a rubber policeman, harvested by centrifugation, and resuspended in lysis buffer (10 mM HEPES; pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 0.2% Nonidet P-40, 50 mM NaF, supplemented with protease inhibitor cocktails). After 30 min of incubation on ice, lysates were clarified by centrifugation (10,000 × g for 20 min at 4°C). For the histone H1 kinase assay, cell lysates (150 μg) was incubated for 2 h at 4°C with anti-cyclin B1 polyclonal antibody (Santa Cruz). Protein A/G beads were then added to the lysates and incubated for 1 h. Immunoprecipitated complexes were washed 3 times with lysis buffer, once with HBS (10 mM HEPES, pH 7.4, 150 mM NaCl). The beads were washed with 5 μg of histone H1 in HBS (20 μl) containing 15 mM MgCl₂, 50 μM ATP, 1 mM dithiothreitol, and 1 μl of [γ-32P]ATP. After 10 and 30 min of incubation at 30°C, the reaction products were analyzed by SDS-PAGE and autoradiography.*

*Subcellular Fractionation—Cell pellets collected from individual dishes were resuspended in lysis buffer (20 μM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.5% Nonidet P-40, 50 mM NaF) supplemented with protease inhibitor cocktails. After 30 min of incubation on ice, lysates were centrifuged at 1000 × g for 10 min at 4°C. The supernatants were cleared again at 13,000 × g for 15 min at 4°C to harvest the cytosolic fraction. The pellets from the first centrifugation were washed twice with lysis buffer and centrifuged again at 1000 × g for 10 min at 4°C. Pellets from this step contained the nuclear fractions.*
**RESULTS**

C53 Was Involved in Modulating Apoptosis Induced by Genotoxic Stress—C53 was originally isolated as a Cdk5 activator p35-binding protein in a yeast two-hybrid screening using p35 as bait (26) and subsequently identified as a caspase substrate. To investigate the cellular function of C53 in regulation of apoptosis, we took the advantage of siRNA technique. As shown in Fig. 1A, C53 siRNA-1 inhibited overexpression of C53-FLAG fusion protein in HeLa cells, and the expression of C53 was examined by immunoblotting. B, stable cell lines with loss of C53 expression. One control and three C53-deficient cell lines were established as described under “Materials and Methods.” Expression of endogenous C53 and various proteins were examined by immunoblotting. V, vector control. C, loss of C53 partially inhibited etoposide-induced cell death. Cells were treated with 20 μM etoposide for 48 h, and the percentage of cell death was scored using trypan blue exclusion assay. Vec, vector. D, loss of C53 partially inhibited both apoptotic and necrotic cell death induced by etoposide. Cells were treated with 20 μM etoposide for 48 h, and cell death was evaluated by annexin V staining and flow cytometry. FITC, fluorescein isothiocyanate. E, ectopic expression of C53 in C53-deficient cells using C53 cDNA with silent mutations. Expression of C53 protein was examined by immunoblotting using anti-FLAG antibody. F, restoration of the sensitivity of C53-deficient cells to etoposide by ectopic expression of C53 (wild-type and the cleavage-defective mutant). Constructs expressing C53 and C53-si (1 μg each) were transiently transfected into HeLa cells along with EGFP (0.2 μg). After 24 h of incubation, cells were treated with etoposide (20 μM) for 48 h. The percentage of cell death was scored by DNA staining of GFP-positive cells. Data were the mean ± S.D., with three independent repeats.

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2 H. Jiang, S. Luo, and H. Li, unpublished data.
affect cell proliferation and cellular morphology.\textsuperscript{3} We further examined the responses of these stable cell lines to DNA topoisomerase II inhibitor etoposide. As illustrated in Fig. 1C, loss of C53 rendered HeLa cells partially but significantly resistant to cell death induced by DNA topoisomerase II inhibitor etoposide. After 48 h of treatment of 20 \mu M etoposide, more than 60% of control HeLa cells underwent cell death, whereas around 30% of C53-deficient cells died. Similar results were observed for E1A-transformed human fibroblast cells IMR90E1A (Supplemental Fig. S1). Moreover, annexin V staining and flow cytometry analysis demonstrated that loss of C53 significantly inhibited both early and late stages of apoptosis induced by etoposide (Fig. 1D), indicating that C53 may modulate etoposide-induced apoptosis.

To further confirm the specificity of C53 shRNA and to test if C53 is indeed required for etoposide-induced apoptosis, we attempted to restore the sensitivity of those stable cell lines to etoposide by expressing C53 ectopically. To avoid inhibition of ectopic expression of C53 protein by C53 shRNA, we introduced two silent mutations in the region of C53 cDNA that were complementary to C53 shRNA. As expected, only the construct of C53-si (C53 cDNA with two silent mutations) could ectopically express wild-type C53 in C53-deficient cells (Fig. 1E), which in turn restored the sensitivity of C53-deficient cell line 6 to etoposide-induced apoptosis (Fig. 1F). Taken together, our results strongly suggest that C53 is likely to play a critical role in modulating etoposide-induced apoptosis.

C53 Was Required for Mitochondrial Damage and Caspase Activation in Etoposide-induced Apoptosis—Because C53 may play a critical role in etoposide-induced apoptosis of HeLa cells, we attempted to understand more about this apoptotic process. Etoposide strongly induced a G2/M arrest in HeLa cells after 24 h of treatment (Fig. 4)\textsuperscript{3} followed by rapid cell death around 40 h post-treatment (Fig. 2A). Meanwhile, a basal level of cell death was also induced by etoposide (20–30% cell death in a 48-h treatment) that is independent of C53 and caspases (Fig. 2, A and B). To investigate the possible working mechanism of C53, we first examined if C53 deficiency resulted in the defects on either drug uptake and/or sensing of DNA damage. As shown in Fig. 2D, loss of C53 did not inhibit up-regulation of p53 induced by etoposide, indicating that C53 may not be involved in drug uptake or sensing of DNA damage. We then

\textsuperscript{3} H. Jiang and H. Li, unpublished data.
Effector of C53 for 24 h, HeLa and cells to etoposide. After transient trans-
combination of phase contrast and fluo-
rescence only, whereas
immunoblotting with indicated antibod-
ies. Total HeLa cell extracts were subjected to
overexpression after etoposide treatment.
caspase activation in the presence of C53
irradiation-induced cell death.
(x-ray irradiation. X-ray irradiation (2.5
staining of GFP-positive cells (DNA
scored by trypan blue assay (cell death). As shown in real-time images (Fig. 3
A), more caspase activation in the presence of C53
DNA staining of GFP-positive cells. Data were the mean ± S.D., with three inde-
dependent repeats. Ctrl, control. C, more caspase
mitochondria (Fig. 2). Activation of both initiator caspases (caspase-2 and -9) and
executioner caspase-3 in etoposide-induced apoptosis of HeLa
cells was partially inhibited by loss of C53 (Fig. 2E), indicating
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C53 acts upstream of mitochondrial damage and caspase activation during etoposide-induced apoptosis.

**Ectopic Expression of C53 Promoted Apoptosis Induced by Genotoxic Stress**—Because C53 deficiency partially inhibited etoposide-induced apoptosis, we further examined if overexpression of C53 could sensitize HeLa cells to genotoxic stress.

As shown in real-time images (Fig. 3A), C53-transfected cells (indicated by fluorescence of EGFP, panel a and b) underwent rapid cell death (as early as 6 h post-treatment, indicated by shrinkage and blebbing) after etoposide treatment, whereas untransfected cells remained in G2/M arrest (indicated by large cell bodies in the center of the images) followed by delayed cell death around 40 h post-treatment (Fig. 2A). After a 24 h treat-
ment, vector-transfected HeLa cells had only 20% cell death, whereas in the presence of C53 overexpression, more than 60% of HeLa cells underwent cell death (Fig. 4B). Similarly, overexpression of C53 also sensitized IMR90E1A cells to etoposide-induced cell death (Fig. 3B and Supplemental Fig. S1B). This result strongly suggests that overexpression of C53 sensitizes tumor cells to etoposide-induced cell death. Furthermore, C53-promoted cell death was apoptotic cell death, as indicated by more poly(ADP-ribose) polymerase cleavage and caspase-3 ac-
tivation in the presence of C53 overexpression (Fig. 3C). Addi-
tionally, we investigated whether C53 is involved in ionizing radiation-induced apoptosis. As shown in Fig. 3, C and D, C53 deficiency partially inhibited x-ray-induced apoptosis of HeLa cells, whereas C53 overexpression promoted it, suggesting that C53 may also play a regulatory role in x-ray-induced apoptosis.

**Ectopic Expression of C53 Rendered HeLa Cells Susceptible to Apoptosis Induced by Multiple Genotoxic Agents in a Cdk1-dependent Manner**—We further examined the effect of C53 on apoptosis induced by other death stimuli. As shown in Supplemental Fig. S2, loss of C53 did not lead to inhibition of apoptosis induced by staurosporine and TNFα as well as other geno-
toxins such as cisplatin and doxorubicin. Nonetheless, overexpression of C53 rendered HeLa cells susceptible to multiple genotoxins such as etoposide, doxorubicin, and camptoth-
ecin, but not cisplatin, TNFα and UV irradiation (Fig. 4A). Because both etoposide and x-ray irradiation normally induce G2/M arrest, the discriminating effect of C53 on sensitization of HeLa cells to apoptosis induced by various agents prompted us to examine cell cycle profiles induced by these agents. Interestingly, flow cytometry analysis showed that treatment of etoposide, doxorubicin, and camptothecin, but not cisplatin, TNFα, and UV radiation induced the G2/M arrest of HeLa cells (Fig. 4B), indicative of a possible correlation between C53 function and apoptosis induced by genotoxins that triggers the G2/M arrest.

Cdk1-cyclin B1 complex is an essential factor for entry and completion of mitosis (4). Previous studies have also demonstrated its regulatory role in apoptotic signaling in a number of experimental paradigms (16). We examined if Cdk1-cyclin B1 is involved in etoposide-induced apoptosis of HeLa cells. Specific Cdk1 inhibitor olomoucine II (IC50 = 20 nM) at the concentration of 2 μM partially inhibited etoposide-induced cell death but had no effect on C53-deficient cells (Fig. 4C). Fur-

**FIG. 3.** Ectopic expression of C53 renders HeLa cells susceptible to eto-
poroside and ionizing radiation. A, real-
time images of C53 overexpressing cells after etoposide treatment. HeLa cells were transfected with C53 construct (1.5 μg) along with EGFP (0.2 μg). After a 24-h incubation, cells were treated with etoposide (20 μM). The real-time images were captured by Leica microscope with Openlab software. Panel a is the green fluorescence only, whereas panel b is the combination of phase contrast and fluorescence images. Panel c and d are phase contrast only. B, ectopic expression of C53 sensitized both HeLa and IMR90E1A cells to etoposide. After transient trans-
fection of C53 for 24 h, HeLa and IMR90E1A cells were treated with 20 and 2 μM etoposide, respectively, for 24 h. The percentage of cell death was scored by DNA staining of GFP-positive cells. Data were the mean ± S.D., with three inde-
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incubation, cells were treated for 24 h with the following agents: etoposide (ETOP; 20 μM), camptothecin (CPT; 2 μM), doxorubicin (Dox; 5 μM), cisplatin (CisP; 10 μM), TNFα (0.5 ng/ml) plus cycloheximide (1 μg/ml), and UV (60 J/m²). NT, no treatment. Cell death was scored by trypan blue exclusion assay. Results were the mean ± S.D., with three independent repeats. A, control.

FIG. 4. Sensitization of HeLa cells by C53 to multiple genotoxins is Cdk1 activity-dependent. A, ectopic expression of C53 renders HeLa cells susceptible to multiple genotoxic agents. HeLa cells were transfected with C53 construct (1.5 μg) along with EGFP (0.2 μg). After 24 h of incubation, cells were treated for 24 h with the following agents: etoposide (ETOP; 20 μM), camptothecin (CPT; 2 μM), doxorubicin (Dox; 5 μM), cisplatin (CisP; 10 μM), TNFα (0.5 ng/ml) plus cycloheximide (1 μg/ml), and UV (60 J/m²). NT, no treatment. Cell death was scored by trypan blue exclusion assay. Results were the mean ± S.D., with three independent repeats. B, G2/M arrest after drug treatment. The percentage of G2/M arrest was evaluated by flow cytometry. Data were the mean ± S.D., with two independent repeats. DMSO, Me2SO. C, Cdk1 inhibitor olomoucine II partially inhibited etoposide-induced cell death of HeLa cells. The control and C53-deficient HeLa cells were treated with 20 μM etoposide for 48 h in the presence of Me2SO or olomoucine II (2 μM). Cell death was scored by trypan blue exclusion assay. Results were the mean ± S.D., with three independent repeats. D, Cdk1 dominant negative mutant inhibited C53-mediated chemosensitization of HeLa cells. HeLa cells were co-transfected with C53 construct and various Cdk DN mutants as indicated. After 24-h of etoposide treatment, cell death was scored by DNA staining of GFP-positive cells. Results were the mean ± S.D., with three independent repeats.

thermore, C53-promoted etoposide-induced cell death was partially inhibited by Cdk1 dominant negative mutant (Cdk1DN), but not by Cdk2DN and Cdk5DN (Fig. 4D). Taken together, these results are suggestive of a critical role of Cdk1 activity in the signaling of etoposide-induced apoptosis of HeLa cells and the sensitization by C53.

Overexpression of C53 Inhibited the Inhibitory Phosphorylation of Cdk1 and Promoted Nuclear Accumulation of Cyclin B1—The result indicative of the involvement of Cdk1 in our experimental system prompted us to examine the effect of C53 on Cdk1-cyclin B1 activity. The 24-h treatment of etoposide did not lead to an increase in overall Cdk1 activity indicated by histone H1 kinase assay (Fig. 5A). However, overexpression of C53 significantly increased Cdk1 activity in the presence of etoposide treatment (Fig. 5A). As described above, activation of Cdk1-cyclin B1 is subjected to multiple levels of transcriptional and post-translational regulation. In response to DNA damage, the G2/M checkpoint system (ATM/ATR and checkpoint kinase 1 and 2) inactivates phosphatase CDC25C, which in turn leads to phosphorylation at residues at Thr-14 and Tyr-15 by kinases Wee1 and Myt1, resulting in inactivation of Cdk1. We then examined the protein level and phosphorylation of Cdk1 and cyclin B1 during the time course of etoposide treatment. As shown in Fig. 5B, the inhibitory phosphorylation of Cdk1 at Tyr-15 was increased when cells were treated with etoposide, indicating proper activation of the G2/M DNA damage checkpoint in HeLa cells. Intriguingly, the inhibitory phosphorylation of Cdk1 at Tyr-15 was dramatically reduced in the presence of C53 overexpression, indicating an increased Cdk1 activity (Fig. 5B). This result was consistent with our Cdk1 kinase assay described in Fig. 5A. Furthermore, the C53-dependent increase on Cdk1 activity was not inhibited by caspase inhibitor zVAD-fmk.2 Taken together, these results indicate that C53 may be involved in regulation of the G2/M DNA damage checkpoint.

We further examined the protein level and phosphorylation of cyclin B1. During the course of etoposide treatment, cyclin B1 accumulated and peaked at 18 h post-treatment and followed by gradual disappearance, presumably due to anaphase-promoting complex-mediated degradation (Fig. 5B). Interestingly, the cyclin B1 level was significantly lower in the presence of C53 overexpression during the time course (Fig. 5B, cyclin B1 panel), whereas its level was higher in C53-deficient cells (Supplemental Fig. S3). To further investigate the role of C53 in regulation of Cdk1-cyclin B1, we first investigated the possible cause of lower cyclin B1 levels in the presence of C53 overexpression. Because C53 overexpression promoted etoposide-induced apoptosis, it is possible that lower cyclin B1 levels may be caused by nonspecific effect of more cell death. To test this possibility, we used pan-caspase inhibitor zVAD-fmk to inhibit most of cell death induced by etoposide. As shown in Fig. 5C, zVAD failed to prevent the decrease of cyclin B1 level induced by C53 overexpression. In contrast, proteasome inhibitor MG132 fully restored cyclin B1 levels in the presence of C53 overexpression. This result suggests that C53 may promote proteasome-mediated cyclin B1 degradation. Because cyclin B1 degradation usually occurs at the exit of mitosis after Cdk1-cyclin B1 activation, the observation of lower cyclin B1 in the presence of C53 overexpression may indirectly indicate that C53 overexpression promotes Cdk1-cyclin B1 activation.

Regulation of the subcellular localization of cyclin B1 is another control mechanism of the G2/M checkpoint. As shown in Fig. 5D, more cyclin B1 were present in the nuclear fraction in the presence of C53 overexpression when cells were treated with etoposide. Moreover, there was more cytoplasmic retention of cyclin B1 in C53-deficient cells (Supplemental Fig. S4A). Phosphorylation of cyclin B1 is required for nuclear accumulation and translocation of Cdk1-cyclin B1 complex from cytoplasm to nucleus during prophase (10, 12–15). C53 overexpres-
Two hundred transcription factors, including but not limited to many promoter sites, are transcribed to DNA damage checkpoint responses. The cellular decision of life versus death involves an intricate network of multiple factors that play critical roles in regulation of DNA repair, cell cycle, and cell death. Among them, multiple checkpoint proteins such as p53, ATM/ATR, etc., play very important roles in cellular decision-making (2). Here we report the involvement of Cdk5 activator-binding protein C53 in regulation of apoptosis induced by genotoxic stress via modulating the G2/M DNA damage checkpoint. Our data demonstrated that C53 deficiency conferred partial resistance to genotoxic agents such as etoposide and x-ray irradiation, whereas ectopic expression of C53 rendered cells susceptible to multiple genotoxins. These results strongly suggest that C53 plays an important role in regulation of genotoxin-induced apoptosis. The finding that C53 sensitized cells only to the genotoxins that trigger G2/M arrest prompted us to examine the effect of C53 on the Cdk1-cyclin B1 complex. Cdk1-specific inhibitors (olomoucine II and Cdk1 DN mutant) inhibited etoposide-induced apoptosis of HeLa cells and C53 sensitizing effect, indicating that Cdk1 alone is not sufficient to initiate apoptotic signaling in our experimental system. Overexpression of C53 enhanced nuclear accumulation and enzymatic activity of Cdk1-cyclin B1, whereas C53 deficiency leads to more cytoplasmic retention of cyclin B1, suggesting that C53 acts as a pivotal player in modulating the Cdk1-cyclin B1 function. Taken together, our results suggest that in response to genotoxic stress, C53 serves as an important regulatory component for the G2/M DNA damage checkpoint.

We found that loss of C53 conferred partial resistance to apoptosis specifically induced by DNA topoisomerase II inhibitor etoposide and x-ray irradiation but not other genotoxic agents. One outstanding question is why loss of C53 confers resistance only to etoposide and x-ray irradiation but not other genotoxins even though the agents such as doxorubicin and camptothecin can also induce G2 arrest and cell death. One possible explanation may lie in the nature of damages produced by genotoxic agents. Most genotoxic agents can produce not only many types of DNA damage but also damages on non-DNA...
targets, which can also trigger responses of cell death. As an example, doxorubicin induces not only DNA damages such as DNA alkylation, cross-linking, and double-stranded breaks but also free radical generation and lipid peroxidation in cellular membrane, mitochondria, and microsomes (27).

In contrast to other genotoxic agents used in this study that can induce HeLa cells to undergo rapid cell death (within 24 h), both etoposide and x-ray irradiation appeared to induce most cells to undergo lengthened G2 arrest (as indicated by the enlarged nucleus and cell body and confirmed by flow cytometry) followed by delayed apoptotic cell death in 48–72 h. Judged by the features of G2 arrest and delayed cell death, etoposide-induced apoptosis of HeLa cells fits well into the definition of “mitotic death” or “reproductive death” (28, 29), a type of cell death usually associated with p53-mutated tumor cells or non-hematopoietic cells as a consequence of DNA damage. Yet the underlying molecular mechanism remains poorly understood. A number of recent studies have indicated that Cdk1 plays a role in regulation of mitotic cell death (16). In this study we observed the increase of Cdk1 activity and nuclear accumulation of Cdk1-cyclin B1 complex when cells were treated with etoposide at late time points. Furthermore, Cdk1-specific inhibitors (Cdk1 DN mutant and pharmacological inhibitor) were able to inhibit etoposide-induced apoptosis. Additionally, our real-time images revealed that typical apoptotic cell death occurred at minutes after cells entered metaphase from G2 phase. Therefore, we postulate that Cdk1 may play a critical role in initiation of mitotic death by driving cells to enter aberrant mitosis, which leads to activation of caspase and apoptosis. If so, what is the link between Cdk1 and the core apoptotic machinery? How do the G2/M checkpoint systems breakdown after lengthened G2 arrest, thereby resulting in Cdk1 activation? Further studies will provide more definitive answers to these important questions.

DNA damage checkpoints are crucial for maintaining DNA integrity in normal cells but also pose as a challenge for conventional cancer treatment. Therefore, abrogation of DNA damage checkpoints appears to be a logical way for chemosensitization of tumor cells. Due to the fact that many cancer cells have defective G1 checkpoints, it becomes more important to modify the G2/M checkpoint for chemosensitization. Our finding that ectopic expression of C53 rendered HeLa cells susceptible to multiple apoptotic stimuli is intriguing with regard to its potential implication on cancer treatment. By overriding the G2/M DNA checkpoint-mediated inhibition of the Cdk1-cyclin B1 activity, ectopic expression of C53 may represent a novel approach for chemo- and radio-sensitization of tumor cells, especially tumor cells with defective G1 checkpoint. Further elucidation of C53 function in both cell cycle and cell death control will facilitate the exploitation of this novel protein in cancer treatment.

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REFERENCES
1. Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002) Annu. Rev. Genet. 36, 617–656
2. Sancar, A., Lindsey-Boltz, L. A., Unaal-Kacmaz, K., and Linn, S. (2004) Annu. Rev. Biochem. 73, 39–85
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3. Zhou, B. B., and Bartek, J. (2004) Nat. Rev. Cancer 4, 216–225
4. Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J. L. (1996) Cell 60, 487–494
5. Lew, D. J., and Kornbluth, S. (1996) Curr. Opin. Cell Biol. 8, 795–804
6. Morgan, D. O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261–291
7. Takizawa, C. G., and Morgan, D. O. (2000) Curr. Opin. Cell Biol. 12, 658–665
8. Sanchez, Y., Wong, C., Thoma, R. S., Shaw, A. S., and Piwnica-Worms, H. (1997) Science 277, 1497–1501
9. Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997) Science 277, 1497–1505
10. Jin, P., Hardy, S., and Morgan, D. O. (1998) J. Cell Biol. 141, 875–885
11. Porter, L. A., and Donoghue, D. J. (2003) Prog. Cell Cycle Res. 5, 335–347
12. Li, J., Meyer, A. N., and Donoghue, D. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 502–507
13. Haggling, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998) EMBO J. 17, 4127–4138
14. Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998) Genes Dev. 12, 2131–2143
15. Hagting, A., Jackman, M., Simpson, K., and Pines, J. (1999) Curr. Biol. 9, 680–689
16. Castedo, M., Perfetti, J. L., Roumier, T., and Kroemer, G. (2002) Cell Death Differ. 9, 1287–1293
17. Zhou, B. B., Li, H., Yuan, J., and Kirschner, M. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6785–6789
18. Shen, S. C., Huang, T. S., Jee, S. H., and Kuo, M. L. (1998) Cell Growth Differ. 9, 23–29
19. Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J., and Hung, M. C. (1998) Mol. Cell 2, 581–591
20. Porter, L. A., Cukier, I. H., and Lee, J. M. (2003) Blood 101, 1928–1933
21. Meinkrantz, W., and Schlegel, R. (1996) J. Biol. Chem. 271, 10205–10209
22. Porter, L. A., Singh, G., and Lee, J. M. (2000) Blood 95, 2645–2650
23. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cell 94, 491–501
24. Li, H., Bergeron, L., Cryns, V., Pasternack, M. S., Zhu, H., Shi, L., Greenberg, A., and Yuan, J. (1997) J. Biol. Chem. 272, 21010–21017
25. Erenpreisa, J., and Cragg, M. S. (2001) Cancer Cell Int. 1, 1–7
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