Ku Affects the Ataxia and Rad 3-related/CHK1-dependent S Phase Checkpoint Response after Camptothecin Treatment

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Abstract

Camptothecin (CPT) that targets DNA topoisomerase I is one of the most promising broad-spectrum anticancer drugs in development today. The cytotoxicity of CPT is S phase (S)-specific because the collision of advancing replication forks with CPT-topoisomerase I-DNA complexes results in DNA damage. After DNA damage, proliferating cells could actively slow down the DNA replication through an S checkpoint to provide time for repair. We report now that there is an activated S checkpoint response in CPT-treated mammalian cells. This response is regulated by Ataxia and Rad3-related (ATR)/CHK1 pathway. Compared with their wild-type counterparts, CPT-treated Ku80−/− cells showed stronger inhibition of DNA replication. This stronger inhibition had no relationship with DNA-dependent protein kinase (DNA-PK) activity but correlated with the higher activities of ATR and the higher activities of CHK1 in such cells. Not only caffeine, the nonspecific inhibitor of ATR, or UCN-01, the nonspecific inhibitor of CHK1, but also the specific CHK1 antisense oligonucleotide abolished the stronger inhibition of DNA replication in CPT-treated Ku80−/− cells. These results in aggregate indicated that the stronger S checkpoint in CPT-treated Ku80−/− cells is regulated through the highly activated ATR/CHK1 pathway.

Introduction

CPT1 is an agent with a unique spectrum of antitumor activity mediated by selective inhibition of DNA Topo I (1, 2). The cytotoxicity of CPT is exerted predominantly during S phase and is associated with a strong inhibition of DNA synthesis (3). Understanding the mechanism of CPT-induced inhibition of DNA replication will allow better control of the cytotoxicity of CPT. It was unclear until recently whether the inhibition was the direct results of a collision of the advancing replication fork with the CPT-Topo I-DNA cleavable complex (3, 4). Our report suggests that the CPT-induced inhibition of DNA replication is not a passive process but is an active one that is the consequence of the S checkpoint activation (5, 6). However, the details of this pathway are unknown.

In response to DNA damage, proliferative cells slow down progression through the S phase, which is the DNA damage-induced S checkpoint. Like the G1 and G2 checkpoint responses, the S checkpoint response is also an active process regulated through the protein kinase pathways (7), which are believed to promote DNA repair and benefit genomic integrity (8). The pathways regulating S checkpoint are conserved in organisms from yeast to human. The mechanism that controls the DNA damage-induced S checkpoint is thought to involve the activations of ATM and ATR kinases (9–12) in mammalian cells. The two pathways regulated by ATM and ATR have some overlapping roles but also have some distinct roles in DNA damage-induced checkpoint regulation (13). However, it remains unclear which kinase pathway, ATM, ATR, or both, regulates S checkpoint response in CPT treated cells.

To extend the study of CPT-induced S checkpoint, we found that the inhibition of DNA synthesis is more apparent in Ku-deficient cells than in their wild-type counterparts. Ku is a multifunction protein containing two subunits, Ku80 and Ku70. Both subunits have a strong ability to bind to double-strand DNA ends and to promote DNA DSBs, nonhomologues end-joining repair, and the process of variable (diversity) joining recombination in mammalian cells (14–18). Ku could also prevent telomere end-joining (19), bind to the origins of DNA replication, and act at the initiation step of DNA replication (20). In this study, we show that the stronger inhibition of DNA synthesis shown in CPT-treated Ku80−/− cells is because of higher activities of ATR/CHK1 kinases. These results provide direct evidence that Ku80 protein could affect ATR activation after CPT treatment and that this effect is linked to ATR-dependent S checkpoint response.

Materials and Methods

Cell Lines, Chemical Treatments, and DNA Synthesis. Ku80 knockout mouse embryonic fibroblasts (14, 21) and DNA-PKcs knockout mouse embryonic fibroblasts (22) were adapted to growing in DMEM supplemented with 10% iron-supplemented calf serum (Sigma Chemical Co.). All of the incubations were at 37°C in an atmosphere of 5% CO2 and 95% air. The DNA synthesis assay was similar to that described before (23). Briefly, cells in the logarithmic phase of growth were prelabeled by culturing in DMEM containing 10 μCi of [3H]thymidine and 0.5 μM cold thymidine for 2 days. This prelabeling provided an internal control for cell number by allowing normalization for total DNA content of samples. The cells were replaced with normal DMEM. Caffeine (Sigma), wortmannin (Sigma), or UCN-01 (National Cancer Institute) was added to the culture for 30 min before CPT (NCS100880, obtained from the National Cancer Institute) treatment. The cells then were treated with CPT for 3 h. [3H]thymidine at 0.1 μCi was added for 30 min. The cells were harvested, loaded onto glass microfiber filters (Whatman GF/A), washed with 10% trichloroacetic acid and deionized water, and incubated for 1 h in 0.5 ml of 0.5 N NaOH at 65°C. Filters were neutralized with HCl, scintillation fluid (Scintiverse; Fisher) was added, and the amount of radioactivity was assayed in a liquid scintillation counter. The rate of DNA synthesis for each sample was calculated as [H] dpm/μCi and is presented as a percentage of the values obtained from nontreated controls.

Flow Cytometry Measurement. As described (24), cells were collected at requested times and fixed in 70% ethanol. Cells were stained with the solution [62 μg/ml RNase A, 40 μg/ml propidium iodide, and 0.1% Triton X-100 in

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3 The abbreviations used are: CPT, camptothecin; Topo I, topoisomerase I; ATM, Ataxia-telangiectasia mutated; S, S phase; GST, glutathione S-transferase; DSB, double-strand break; ATR, Ataxia and Rad3-related; DNA-PKcs, DNA-PK catalytic subunit; RIPA, radioimmunoprecipitation assay; PMSF, phenylmethylsulfonyl fluoride.
PBS buffer, 137 mM NaCl, 2.7 mM KCl, 10 μM Na₂HPO₄, 2 μM KH₂PO₄ at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite).

*ATR Western Blot and Kinase Activity Assay.* Nuclear extracts were prepared by using the NE-PER kit (Pierce) according to the manufacturer's instruction. The fractions of chromatin-bound extract were prepared as described previously (25). Briefly, cells were collected and washed in cold PBS. Proteins were then extracted with cold 0.1% Triton X-100 in CSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF) for 20 min at 4°C. The chromatin-bound fraction was then pelleted by low-speed centrifugation at 3,000 rpm for 5 min at 4°C. The supernatant was named fraction 1. This supernatant was then re-extracted by incubating in CSK buffer and collected by centrifugation at 16,000 rpm for 10 min at 4°C. This supernatant was named fraction 2. The final pellet fraction (containing chromatin-bound proteins) was solubilized in RIPA buffer [150 mM NaCl, 40 mM 4-morpholinepropanesulfonic acid (pH 7.2), 1 mM EDTA, 1% NP40, 1% sodium deoxycholate, and 0.1% SDS] and was named fraction 3. For kinase assay, 500 μg of nuclear extracts or fraction 3 was then mixed with 2 μg of ATR antibody in the presence of 20 μl of a 50% (v/v) protein G-Sepharose slurry (Life Technologies, Inc.) in 500 μl of Buffer A (0.5% NP40, 1 mM Na₂VO₄, 5 mM NaF, and 0.2 mM PMSF in PBS buffer) and gently rotated overnight at 4°C. Immune complexes were washed twice with Buffer A and then twice with Buffer B [100 mM HEPES (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM DTT]. The kinase immunoprecipitate was incubated at 30°C for 30 min with 1 μg of phosphorylated heat- and acid-stable protein regulated by insulin (Stratagene) in 25 μl Buffer B containing 10 μCi [(γ-³²P)ATP. Samples were analyzed by 12% SDS-PAGE and the kinase activities determined by the incorporation of ³²P into phosphorylated acid-stable protein regulated by insulin protein using the PhosphorImager. The naked chromatin-bound proteins were prepared by modifying the method with the NE-PER kit (Pierce). Briefly, 2 × 10⁶ cells were collected for each sample preparation. The preparations of cells lysis and cytoplasmic extracts were according to the manufacturer’s instruction. After the pellets were suspended in nuclear extract buffer provided in the kit, samples were vortexed at the highest speed for 15 s, kept in ice for 10 min, centrifuged at 16,000 rpm for 10 min, the supernatant (nuclear extract) fractions were then mixed with 2 μg of ATR antibody in the presence of 20 μl of a 50% (v/v) protein G-Sepharose slurry (ReplGen) in 250 μl of Buffer A (0.5% NP40, 1 mM Na₂VO₄, 5 mM NaF, and 0.2 mM PMSF in PBS buffer) and gently rotated overnight at 4°C. The immune complexes were washed as described above. The kinase immunoprecipitate was incubated at 30°C for 30 min with 1 μg of GST-CDC25C (200–256) in 25 μl Buffer B containing 5 μCi [(γ-³²P)ATP. Samples were analyzed by 12% SDS-PAGE and the kinase activities determined by the incorporation of ³²P into CDC25C protein using the PhosphorImager.

**Purification of GST-CDC25C.** BL21 cells were transformed with plasmid encoding GST-CDC25C[200–256]. The GST-CDC25C was purified by using the microspin GST purification module kit (Amersham Pharmacia Biotech Inc.) according to the manufacturer’s instructions.

**CHK1 Kinase Activity Assay.** For this purpose, cell extracts were prepared by using NE-PER kit (Pierce) according to the manufacturer’s instruction. Nuclear extracts (250 μg) were then mixed with 1 μg of CHK1 antibody (sc-7898; Santa Cruz) in the presence of 10 μl of a 50% (v/v) protein A-Sepharose slurry (ReplGen) in 250 μl of Buffer A (0.5% NP40, 1 mM Na₂VO₄, 5 mM NaF, and 0.2 mM PMSF in PBS buffer) and gently rotated overnight at 4°C. The immune complexes were washed as described above. The kinase immunoprecipitate was incubated at 30°C for 30 min with 1 μg of GST-CDC25C[200–256] in 25 μl Buffer B containing 5 μCi [(γ-³²P)ATP. Samples were analyzed by 12% SDS-PAGE and the kinase activities determined by the incorporation of ³²P into CDC25C protein using the PhosphorImager.

**Purification of Recombinant Ku70/Ku80 Heterodimer from Insect Cells.** Human genes encoding full-length Ku70 and Ku80 were cloned into the baculoviral shuttle vector pVL1393 (Invitrogen). Recombinant Ku70/Ku80 was purified as described previously (26).

**Treatment of Cells with Oligonucleotides.** The antisense oligonucleotide of Chk1 (5’-ggcaacgccagcatgca-3’) was designed to specifically target the sequence of the start codon region of Chk1 mRNA (24). The sense oligonucleotide of human ATM (5’-ACCATGAATCAGACCTC-3’) acted as the control. The oligonucleotides were delivered to cells by OligoFECTAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Briefly, antisense oligonucleotides (0.5 μM) were added to serum-free MEM containing 20 μg/ml OligoFECTAMINE reagent. This preparation (1 ml) was added to 30% confluent cells cultured in 60-mm plate (prelabeled with 10 μCi [³⁵S]thymidine for 24 h) for 4.5 h. Additional DMEM (0.5 ml) with 30% iron-supplemented calf serum was then added to the culture for another 24 h. The cells either were prepared to measure the inhibition of DNA synthesis 3 h after CPT treatment as described above or were directly collected in 1× protein loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol] and prepared for Western Blot. Antibodies of CHK1 (sc-8404; Santa Cruz) and glyceraldehyde-3-phosphate dehydrogenase (Chemicon International) were used in this experiment.

**Results**

**Stronger S Checkpoint Response Was Shown in CPT-treated Ku80⁻/⁻ Cells.** S checkpoint response is measured as a transient decrease in [³²P]thymidine incorporation (9, 10) after CPT treatment. Compared with their wild-type counterparts, Ku80⁻/⁻ cells showed a stronger inhibition of DNA synthesis after CPT treatment (Fig. 1, A and B), which reflected the stronger S checkpoint response in CPT-treated Ku80⁻/⁻ cells. Ku80 is one subunit of the trimer protein, DNA-PK, which also includes Ku70 and DNA-PKcs. Binding of Ku to DSB DNA ends recruited and activated DNA-PKcs. Therefore, without Ku80, the cells have no DNA-PK kinase activity. To distinguish that the stronger S checkpoint response in Ku80⁻/⁻ cells was directly because of Ku or was indirectly because of DNA-PK activity, we then examined the DNA synthesis in CPT-treated DNA-PKcs knockout cells. There was no apparent difference in the rates of DNA synthesis between DNA-PKcs⁻/⁻ and DNA-PKcs⁻/- cells (data not shown), which suggested that Ku80 directly affected the stronger S checkpoint response after CPT treatment.

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Fig. 1. Stronger S checkpoint responses are shown in CPT-treated Ku80⁻/⁻ cells. A, DNA synthesis was examined at 3 h after various doses of CPT treatment in Ku80⁻/⁻ and Ku80⁺/⁺ cells. The rate of DNA synthesis was calculated as [³⁵S]dpm/³¹C dpm for the various samples and was presented as percentage of the values obtained from nontreated cells. B, DNA synthesis was examined 3 h after 1 μM CPT treatment in Ku80⁻/⁻ and Ku80⁺/⁺ cells. Caffeine (4 μM), wortmannin (10 μM), and UCN-01 (100 μM) were added to the cultures 30 min before CPT treatment. The rate of DNA synthesis in cells treated with inhibitor plus CPT was presented as percentage of the values obtained from cells treated with inhibitor alone. Data shown are the average from three independent experiments. 

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The Stronger S Checkpoint Response in Ku80⁻/⁻ Cells Was Sensitive to Caffeine and UCN-01. We reported that the DNA damage-induced S checkpoint could be regulated by two different but correlated pathways: ATM-dependent and ATR/CHK1 dependent (12). To investigate which pathway is responsible for the stronger S checkpoint response in Ku80⁻/⁻ cells, we examined the effects of different kinase inhibitors (caffeine, wortmannin, and UCN-01) on the rate of DNA synthesis in CPT-treated Ku cells. Wortmannin (10 μM) did not change the rate of DNA synthesis in CPT-treated Ku cells, but both caffeine (4 mM) and UCN-01 (100 nM) dramatically increased the rate of DNA synthesis in CPT-treated Ku cells. The difference in the DNA synthesis rates between CPT-treated Ku80⁺/⁺ and Ku80⁻/⁻ cells disappeared (Fig. 1B). Neither caffeine nor UCN-01 alone apparently changed S ratio of these cells at 3 h after CPT treatment (Fig. 1C), indicating that the recovered DNA replication in CPT-treated cells by caffeine or UCN-01 is not because of less S cells in caffeine or UCN-01 alone treated cells. Caffeine (4 mM) inhibits ATM and ATR activities but does not affect DNA-PK activity (27). Wortmannin (10 μM) inhibits ATM and DNA-PK activities but does not affect ATR activity (27). UCN-01 (100 nM) inhibits CHK1, the downstream target of ATR, but does not affect CHK2, the downstream target of ATM (28, 29). These data about inhibitors indicated that CPT-induced S checkpoint response is mainly regulated by a wortmannin-resistant but caffeine- or UCN-01-sensitive pathway, which might be an ATM-independent and ATR/CHK1-related pathway. The stronger S checkpoint response shown in Ku80⁻/⁻ cells suggested that Ku80 is involved in S checkpoint regulation in CPT-treated cells.

Higher ATR Activity Accompanied with More ATR Bound to Chromatin DNA in CPT-treated Ku80⁻/⁻ Cells. To investigate whether ATR activation was responsible for the stronger S checkpoint response in CPT-treated Ku80⁻/⁻ cells, we next examined the ATR activity in Ku cells. We first measured the ATR protein levels in the nuclear extracts (prepared by using the NE-PER kit) of Ku cells either with or without CPT treatment. We found that the ATR levels were lower in the nuclear extracts of CPT-treated Ku cells than that in control Ku cells (Fig. 2A, NE, and Fig. 2B) although the ATR activity did not show any apparent differences (Fig. 2A, NE), suggesting that higher ATR activity with equal amount of such protein existed in CPT-treated Ku80⁻/⁻ cells. We then measured ATR levels in chromatin-bound extracts (prepared as described in “Materials and Methods”). Although the levels of ATR in this fraction of extracts derived from the control cells were similar to those found in the CPT-treated cells (Fig. 2A, CHE), ATR activity of this fraction in CPT-treated Ku cells was higher than shown in nontreated control Ku cells, and the increased level was more apparent in Ku80⁻/⁻ cells than in Ku80⁺/⁺ cells (Fig. 2A, CHE, and Fig. 2C). These results indicated that ATR activity increased in CPT-treated cells. Without Ku, ATR activity increased even higher after CPT treatment, suggesting involvement of Ku in ATR activation.

To investigate whether Ku directly inhibits ATR activity, we compared the ATR activity of Ku80⁻/⁻ cells with and without additional purified Ku proteins in the in vitro assay. There was no difference in ATR activity from Ku80⁻/⁻ cells with and without additional purified Ku in such reactions (data not shown), which suggested that Ku80 does not inhibit ATR activity directly and suggested that Ku80 affecting ATR activity might be through other indirect ways. ATR is a chromatin-binding protein (30). To test whether higher ATR activity shown in CPT-treated Ku80⁻/⁻ cells was attributable to more ATR bound to chromosome DNA, we also measured ATR levels in naked chromatin-bound extracts because the regular chromatin-bound extracts prepared as described in “Materials and Methods” still contained many proteins that did not really bind to chromatin DNA. The results are shown in Fig. 2D. There is a barely detectable signal of ATR (Ku80⁺/⁺ and Ku80⁻/⁻ cells) and Ku (Ku80⁺/⁺ cells) only in the naked chromatin-bound fraction from non-treated control cells. There are clear signals of both ATR (Ku80⁺/⁺ and Ku80⁻/⁻ cells) and Ku (Ku80⁻/⁻ cells) only in the fraction from CPT-treated cells (Fig. 2D), suggesting that ATR and Ku bind to the site of CPT-induced DNA damage. More ATR signal was shown in the naked chromatin-bound fraction from CPT-treated Ku80⁻/⁻ cells than from the CPT-treated Ku80⁺/⁺ cells, suggesting the effect of Ku protein on such ATR-binding process.

Higher CHK1 Activity Was Shown in CPT-treated Ku80⁻/⁻ Cells. CHK1 is one downstream target of ATR (31). To test whether the higher ATR activity shown in CPT-treated Ku80⁻/⁻ cells was through activating CHK1 pathway, we examined the CHK1 activity in Ku cells. The protein levels are the same in cytoplasmic extracts from Ku80⁺/⁺ and Ku80⁻/⁻ cells only) in the fraction from CPT-treated cells (Fig. 2D), suggesting that ATR and Ku bind to the site of CPT-induced DNA damage. More ATR signal was shown in the naked chromatin-bound fraction from CPT-treated Ku80⁻/⁻ cells than from the CPT-treated Ku80⁺/⁺ cells, suggesting the effect of Ku protein on such ATR-binding process.

CHK2 is another important S checkpoint regulator (11). To study whether CHK2 was also involved in the stronger S checkpoint response in CPT-treated Ku80⁻/⁻ cells, we measured CHK2 activity in

![Figure 2](cancerres.aacrjournals.org)
Results obtained by quantitating gels from three independent experiments bars, B. CHK1 measured with the nuclear extracts (quantitative analysis of the data shown in "Materials and Methods." B. CHK1 activities were measured with the nuclear extracts (NE). C, quantitative analysis of the data shown in B. Results obtained by quantitating gels from three independent experiments bars, ±SD.

CPT-treated Ku cells. Although ~15% of CHK2 activity increased in CPT-treated cells compared with that in control cells, there was no difference between Ku80+/+ and Ku80−/− cells (data not shown). UCN-01, which inhibits CHK1 without affecting CHK2 (28, 29), almost completely recovered the inhibition of DNA replication in CPT-treated cells, suggesting that CHK2 is not the major regulator in CPT-induced S checkpoint response.

Down Expression of Chk1 Abolished the Stronger S Checkpoint Response in CPT-treated Ku80−/− Cells. To additionally determine whether the higher activity of CHK1 was responsible for the stronger S checkpoint response in CPT-treated Ku80−/− cells, we examined the effects of Chk1 antisense oligonucleotides on the DNA synthesis of CPT-treated Ku cells. The results are shown in Fig. 4. The Chk1 antisense oligonucleotides specifically inhibited Chk1 expression in both Ku80+/+ and Ku80−/− cells (Fig. 4A) and abolished the inhibition of DNA synthesis in CPT-treated Ku cells, which resulted in similar levels of DNA synthesis in Ku80+/+ and Ku80−/− cells (Fig. 4B). These results demonstrated that CHK1 played a key role in the S checkpoint response of CPT-treated Ku cells and indicated that the stronger S checkpoint response in CPT-treated Ku80−/− cells was because of a highly activated ATR/CHK1 pathway.

Discussion

S Checkpoint Signals in CPT-treated Cells. The target of CPT is DNA Topo I (1, 2). CPT could induce DNA DSBs through the collision of the advancing replication fork with the CPT-Topo I-DNA cleavable complex (3, 4). Either the DNA DSBs or the block of the replication fork could activate the S checkpoint. The former (DNA damage checkpoint) immediately activates the ATM-dependent pathway then activates the ATR-dependent pathway (9, 10, 12) and the latter (replication checkpoint) mainly activates the ATR-dependent pathway (30). What signal activates the S checkpoint in CPT-treated cells? We showed previously that (6) the inhibition of DNA synthesis in the CPT-treated ATM wild-type cells was reversed to a small extent by wortmannin, the nonspecific inhibitor of ATM (27), and indicated that ATM is a partial factor affecting the CPT-induced S checkpoint response, and suggested that there is another major factor, which is ATM-independent and caffeine sensitive, regulating the S checkpoint in CPT-treated cells (6). Here, the results show that wortmannin did not affect the rate of DNA replication in CPT-treated Ku cells. The different effects of wortmannin on these cells might be because of the different lines derived from different species. Similar to the previous report, we show that caffeine, the nonspecific inhibitor of ATM and ATR, completely released the inhibition in all types of CPT-treated cells. Although DNA DSBs exist in CPT-treated cells, the amount of DSBs in 2-μM CPT-treated (for 3 h) cells that showed the strong inhibition of DNA synthesis is roughly equal to that in 5 Gy irradiated cells (5) in which the inhibition is very weak. These results support the explanation that the ATR-dependent pathway is the major regulator of the S checkpoint in CPT-treated cells, which suggests that block of the replication fork by the CPT-Topo I-DNA cleavable complex rather than the DSBs is the major signal to activate ATR kinase.

Ku Affecting the Activation ATR in CPT-treated Cells. ATR is a chromatin-binding protein and is required for the S checkpoint induced by aphidicolin (30). ATR activation induced by aphidicolin correlates with an accumulation of ATR on chromatin DNA (30). Our data show that more ATR bound to chromatin DNA correlates with higher activity of ATR in CPT-treated cells. Without Ku, even more ATR bound to chromatin DNA after CPT treatment suggests that Ku affects such binding. Higher activity of ATR was observed in CPT-treated Ku80−/− cells, suggesting that ATR binding to the sites of DNA damage induced by CPT is a prerequisite for its activation. Because Ku also is a replication-dependent, chromatin-binding protein (20), the mechanism by which Ku affects the activation of ATR in CPT-treated cells might be attributable to the competition between these two proteins binding to DNA. However, more studies are required to obtain proof of this conjecture. Although Ku does not inhibit ATR activity in vitro, we cannot exclude the possibility that Ku directly inhibits ATR activity in vivo.

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ATR/CHK1 Pathway in CPT-induced S Checkpoint. Several observations suggest that CHK1 is a downstream target of ATR (32-34). ATR-mediated checkpoint pathways regulate phosphorylation and activation of CHK1 (31). It was reported that the phosphorylation was observed in CPT-treated yeast cells (35), but our Western Blot results do not show the phosphorylation bands of CHK1 in CPT-treated cells, which might be because of the different sensitivities of the phosphorylation in the different species, and the regular one-dimensional gel may not be sensitive enough to observe the phosphorylation of mammalian CHK1 (32). Our results indicate that CHK1 is activated in CPT-treated cells. Higher activity was also observed in the nuclear extracts than in the cytoplasmic extracts of CPT-treated Ku cells with the same amount of protein (data not shown), which suggests that the activity of CHK1 depends on its conformation. The facts that Ku80<sup>−/−</sup> cells show even higher activity of CHK1 than Ku80<sup>−/−</sup> cells after CPT treatment is consistent with the ATR data, which suggests that the conformation change of CHK1 might be the phosphorylation of CHK1 by ATR. CHK1 antisense oligonucleotide abolishes the stronger S checkpoint response in CPT-treated Ku80<sup>−/−</sup> cells, which gives direct evidence that the ATR/CHK1 pathway plays the key role in the S checkpoint regulation. CHK1 expression is cell cycle dependent (36); therefore, its protein level might be critical for its function. We reported previously that A1–5, one transformed rat embryo fibroblast cell line, with a higher CHK1 level showed much stronger checkpoint response than its counterparts (24), supporting this hypothesis.

In summary, our results demonstrate that CPT-induced inhibition of DNA replication reflects an active S checkpoint response in mammalian cells, which is mainly regulated by the ATR/CHK1 pathway. The strongest inhibition of DNA replication shown in CPT-treated Ku80<sup>−/−</sup> cells is attributable to higher activities of ATR/CHK1 kinases, indicating that Ku protein affects ATR regulated S checkpoint activation.

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3. Hsiang, Y.-H., Lihou, M. G., and Liu, L. F. Arrest of replication forks by drug-resistant mutant human tumor cells after CPT treatment is consistent with the ATR/CHK1 pathway. Several observations suggest that CHK1 is a downstream target of ATR (32-34). ATR-mediated checkpoint pathways regulate phosphorylation and activation of CHK1 (31). It was reported that the phosphorylation was observed in CPT-treated yeast cells (35), but our Western Blot results do not show the phosphorylation bands of CHK1 in CPT-treated cells, which might be because of the different sensitivities of the phosphorylation in the different species, and the regular one-dimensional gel may not be sensitive enough to observe the phosphorylation of mammalian CHK1 (32). Our results indicate that CHK1 is activated in CPT-treated cells. Higher activity was also observed in the nuclear extracts than in the cytoplasmic extracts of CPT-treated Ku cells with the same amount of protein (data not shown), which suggests that the activity of CHK1 depends on its conformation. The facts that Ku80<sup>−/−</sup> cells show even higher activity of CHK1 than Ku80<sup>−/−</sup> cells after CPT treatment is consistent with the ATR data, which suggests that the conformation change of CHK1 might be the phosphorylation of CHK1 by ATR. CHK1 antisense oligonucleotide abolishes the stronger S checkpoint response in CPT-treated Ku80<sup>−/−</sup> cells, which gives direct evidence that the ATR/CHK1 pathway plays the key role in the S checkpoint regulation. CHK1 expression is cell cycle dependent (36); therefore, its protein level might be critical for its function. We reported previously that A1–5, one transformed rat embryo fibroblast cell line, with a higher CHK1 level showed much stronger checkpoint response than its counterparts (24), supporting this hypothesis.

In summary, our results demonstrate that CPT-induced inhibition of DNA replication reflects an active S checkpoint response in mammalian cells, which is mainly regulated by the ATR/CHK1 pathway. The strongest inhibition of DNA replication shown in CPT-treated Ku80<sup>−/−</sup> cells is attributable to higher activities of ATR/CHK1 kinases, indicating that Ku protein affects ATR regulated S checkpoint activation.

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