Chk1 Mediates S and G2 Arrests through Cdc25A Degradation in Response to DNA-damaging Agents*

Zhan Xiao‡, Zehan Chen, Angelo H. Gunasekera, Thomas J. Sowin, Saul H. Rosenberg, Steve Fesik, and Haiying Zhang§

From Cancer Research, Abbott Laboratories, Abbott Park, Illinois 60064-6101

UV and ionizing radiation (IR) activate DNA damage checkpoints and induce Cdc25A degradation (Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000) Science 288, 1425–1429; Falck, J., Mailand, N., Syljuasen, R. G., Bartek, J., and Lukas J. (2001) Nature 410, 842–847). The degradation of Cdc25A is abrogated by caffeine, which implicates Chk1 as the potential mediator (Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000) Science 288, 1425-1429). However, the involvement of Chk1 is far from clear, because caffeine is a rather nonspecific inhibitor of the ATR/Chk1 signaling pathway. Additionally, it is not known whether DNA-damaging drugs commonly used in chemotherapy, which may activate different signal transduction pathways than UV or IR, also confer Cdc25A degradation. Herein, we show that camptothecin and doxorubicin, two widely used topoisomerase inhibitors conferring S and G2 arrest, respectively, cause the degradation of Cdc25A. Using a small interfering RNA that enables the specific elimination of Chk1 expression, we show that the observed proteolysis of Cdc25A is mediated through Chk1. Moreover, Cdc25A overexpression abrogates the Chk1-mediated degradation and overcomes the doxorubicin-induced G2 arrest through dephosphorylation and activation of Cdc2/Cdk1 in a dose-dependent manner. These results suggest that: (a) Cdc25A is involved in the G2/M transition in addition to its commonly accepted effect on G1/S progression, and (b) Chk1 mediates both S and G2 checkpoint and is thus a more ubiquitous cell cycle checkpoint mediator than previously thought.

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DNA damage caused by different agents induces cell cycle arrest at G1, S, or G2 thereby preventing replication of damaged DNA or aberrant mitosis until the damage is adequately repaired. These regulatory mechanisms are known as cell cycle checkpoints and involve an intricate network of protein kinase signaling pathways. They are central to the maintenance of genomic integrity and basic viability of the cells. Hence, defects in these pathways may result in either tumorigenesis or apoptosis depending on the severity and particular nature of the defects (3, 4).

Chk11 is a major checkpoint kinase and has been shown to be

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† To whom requests for reprints should be addressed. E-mail: zhan.xiao@abbott.com.
‡ To whom correspondence should be addressed. Tel.: 847-938-4857; Fax: 847-935-7551; E-mail: haiying.zhang@abbott.com.
§ The abbreviations used are: Chk1, checkpoint kinase 1; CDK, cyclin-dependent kinases; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; BrdUrd, bromodeoxyuridine; scrRNA, scrambled control RNA; siRNA, small interfering RNA; AT, ataxia telangiectasia; ATR, ataxia and Rad3 related.
role in the DNA damage-induced G2 checkpoint, Chk1 has become an attractive target for this approach, and Chk1 inhibitors are being developed to potentiate the effects of genotoxic chemotherapeutics on p53-null tumors.

Herein we demonstrate that camptothecin and doxorubicin, two DNA-damaging agents that cause S or G2 arrest, respectively, activate Chk1 and cause the rapid proteolysis of Cdc25A. Elimination of Chk1 expression through siRNA not only abrogated the S or G2 arrest, but also protected Cdc25A from degradation. These results suggest that Chk1 mediates the S or G2 phase checkpoints by targeting Cdc25A for proteolysis following chemically induced DNA damage. This study indicates that a Chk1 inhibitor may potentiate the cytotoxicity not only of DNA-damaging drugs causing G2 arrest, but also of agents conferring S arrest, which significantly broadens its applicability to cancer chemotherapy. Our results additionally demonstrate that Cdc25A plays functional roles not only in the G2/S transition but also in G2/M progression. This later conclusion is supported by our observation that both Cdc25A and Cdc25C overexpression can overcome the Chk1-mediated G2/M arrest induced by doxorubicin in a dose-dependent manner.

**MATERIALS AND METHODS**

**Cell Culture**—Human lung cancer cell line H1299 was obtained from ATCC (Manassas, VA). H1299 were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.45% glucose at 37 °C in a 5% CO2 incubator.

**Drug Treatment**—Cdc25A was detected with NeoMarker monoclonal anti-cdc25A antibody C-3 (Lab Vision, CA). Chk1 was detected with Chk1 (G-4) monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Cdc25C was detected by Cdc25C (C-20) polyclonal antibody from Santa Cruz Biotechnology. Phospho-Chk1 (Ser-345) was detected by phospho-Chk1 (Ser-345) antibody from Cell Signaling Technology.

**Transfection**—Human Chk1 siRNA and scrambled control siRNA were designed according to the recommendations of the manufacturer (Dharmacon Research). Chk1 siRNA oligonucleotide contains the following sequence: aacaagtgaagcagtcgcagt. The scrambled siRNA derived from Chk1 siRNA contains sequence: aactgaagaagcagtcgcagt. The scrambled siRNA derived from Chk1 siRNA contains sequence: aacaagtgaagcagtcgcagt.

For siRNA transfection alone, H1299 cells were seeded at 2.5 × 10⁶ cells/well into a 6-well plate with 2 ml of medium in each well. The next day, the cells were transfected with siRNA oligonucleotides with Oligofectamine (Invitrogen, Carlsbad, CA) according to the vendor’s instructions with slight modifications. Briefly, 10 μl of siRNA at 20 μM was mixed with 140 μl of Opti-MEM (Invitrogen) to obtain solution A. In solution B, 10 μl of Oligofectamine was added to 40 μl of Opti-MEM and incubated at room temperature for 10 min. Then, solutions A and B were mixed together and allowed to incubate for 20–30 min and then added to each well containing 2 ml of the medium in a 6-well plate.

For co-transfection of Chk1 siRNA with Cdc25A expression plasmid, 10 μl of siRNA with 0.1 μg of the plasmid were added to 0.1 ml of Opti-MEM to obtain solution A. In solution B, 5 μl of Lipofectamine 2000 (Invitrogen) was added to 100 μl of Opti-MEM and incubated at room temperature for 10 min. Then, solutions A and B were combined, allowed to incubate for 20–30 min, and then added to each well.

**Drug Treatment**—DNA-damaging agent-induced Cdc25A degradation was carried out with the following treatment regimens: for camptothecin, either 3 μM for 3–6 h or 0.2–0.3 μM for 18–24 h (both achieved similar results); for doxorubicin, 0.5 μM for 18–24 h.

**Western Blot Analysis**— Cultured cells were rinsed with PBS and directly lysed in Laemmli sample buffer supplemented with 0.5% β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μM activated sodium orthovanadate, 200 ng okadaic acid, and 1× protease inhibitor cocktail (Roche Diagnostics). Lysed samples were treated at 95 °C for 5 min and resolved on the Novex mini-gel system (Invitrogen) under denaturing conditions and blotted to polyvinylidene difluoride membrane using a semi-dry transfer device (Amersham Biosciences). The membrane was blocked with 5% nonfat dry milk and probed with antibodies. Chemiluminescent detection was performed using ECL reagents according to the vendor’s protocols (Santa Cruz Biotechnology).

**RESULTS**

**Camptothecin Induces Phosphorylation of Chk1 and Cdc25A Degradation, Resulting in S Phase Arrest**—It was previously reported that Cdc25A undergoes proteolysis upon IR or UV irradiation (1, 2), which induce G2 or G1 arrest, respectively. To investigate if this finding can be extended to DNA damage-induced arrest at other phases of the cell cycle, we studied the effect of camptothecin, a clinically relevant topoisomerase I inhibitor conferring S phase arrest. H1299 cells, a p53-null lung cancer cell line, were treated with or without camptothecin and harvested for Western blot analysis of Cdc25A, Chk1 protein levels, and cell cycle profiles. Cdc25A almost completely disappeared after the treatment (Fig. 1A, compare lanes 3 and 4 versus 1 and 2), whereas its close homologue, Cdc25C, did not show any change in protein levels. The disappearance of Cdc25A is because of proteasome-mediated degradation, because incubation of cells with MG132, a specific proteasome inhibitor, abrogated this phenomenon (data not shown). Although the amount of Chk1 protein remained constant before and after drug treatment, the ATR-phosphorylated Chk1 species (Chk1 Ser345) appeared only after camptothecin addition (lanes 3 and 4), indicating Chk1 activation in response to camptothecin-induced DNA damage. We additionally confirmed that cells treated with camptothecin were mostly arrested in S phase (Fig. 2B and data not shown), further suggesting that Chk1 may mediate S checkpoint by targeting Cdc25A for proteolysis.

**Doxorubicin Induces Phosphorylation of Chk1 and Cdc25A Degradation, Resulting in G2/M Arrest**—We also studied the effect of doxorubicin, a clinically relevant topoisomerase II inhibitor conferring G2 arrest on different cancer cell lines including H1299 cells (Fig. 3A and data not shown). Similar to camptothecin, doxorubicin caused rapid degradation of Cdc25A while leaving Cdc25C mostly intact, which can be correlated to the phosphorylation and activation of Chk1 upon drug treatment (Fig. 1B). Thus, different DNA-damaging agents, which cause arrests at different phases of the cell cycle, lead to simultaneous Chk1 activation and Cdc25A degradation. These observations are not specific to H1299 cells, because similar findings were also obtained from other cell lines including SW620 and HCT-15 (data not shown).

**Chk1 Mediates the Proteolysis of Cdc25A in Response to Camptothecin and Doxorubicin**—To confirm the direct involvement of Chk1 in the DNA damage-induced proteolysis of Cdc25A, we designed an siRNA that specifically eliminated Chk1 expression. Chk1 siRNA or the scrambled control RNA
(scrRNA) were transfected into H1299 cells that were subsequently subjected to the same camptothecin treatment (Fig. 2A). For scrRNA, we again observed the efficient proteolysis of Cdc25A in response to camptothecin at both 3- and 6-h time points (lanes 1–3). In contrast, with transfection of the Chk1 siRNA, the proteolysis of Cdc25A was significantly inhibited (lanes 4–6). The identity of the indicated Cdc25A band was confirmed by its exact co-migration with overexpressed Cdc25A protein (data not shown; also see Fig. 2B). The Chk1 immunoblot confirms the effective elimination of the Chk1 protein by the Chk1 siRNA but not the scrRNA. This result established that Chk1 mediates the degradation of endogenous Cdc25A in response to camptothecin treatment.

To further confirm this observation, we ectopically expressed Cdc25A in H1299 cells. When Cdc25A is moderately overexpressed (about 3–5-fold over endogenous level), camptothecin still induces the efficient proteolysis of Cdc25A (Fig. 2B, lanes 1–3). However, with Chk1 siRNA, this degradation was essentially abrogated (lanes 4–6). In fact, the steady-state level of Cdc25A was greatly increased (by 3–5-fold, compare lanes 1 and 4), indicating that under these conditions Chk1 also negatively regulates the level of Cdc25A even in the absence of any DNA damage.

To ascertain whether Chk1 also directly mediates doxorubicin-induced Cdc25A degradation, we treated H1299 cells with doxorubicin at 500 nM overnight to induce the proteolysis of Cdc25A. With Chk1 scrRNA transfection, this process remains intact (Fig. 2C, lanes 3 and 4). In contrast, the Chk1 siRNA greatly inhibited this clearance (lanes 1 and 2). These results suggest that Chk1 targets Cdc25A for degradation in response to both doxorubicin and camptothecin.

**Chk1 Mediates Both S and G2 Checkpoints**—To investigate whether Chk1 is involved in the doxorubicin-induced G2 checkpoint, we analyzed the cell cycle profile of H1299 cells in the presence of doxorubicin with or without the elimination of Chk1 via siRNA. When cells transfected with scrRNA were treated with doxorubicin for 24 h, we observed a dramatic increase in the G2/M peak (19–79%), indicating the induction of the G2 checkpoint (Fig. 3A, profiles 1 and 2). However, when Chk1 expression is eliminated via the Chk1-specific siRNA, the extent of G2/M arrest was significantly reduced (28%, Fig. 3A, profile 4), indicating that Chk1 is directly responsible for the doxorubicin-conferring G2 checkpoint. Additionally, we observed a large increase of apoptotic (M1 phase, sub-G0/G1) cells.

**Fig. 1.** Both camptothecin and doxorubicin induce Cdc25A degradation. A, camptothecin induces Cdc25A degradation in H1299 cells. H1299 cells were treated with or without 1 μM camptothecin for 4 h. Cells were washed with PBS and directly lysed in SDS sample buffer. Protein lysates were immunoblotted for Cdc25A, Chk1, phosphorylated Chk1 (P-Chk1 S345P), Cdc25C, and actin (for loading control). Samples were prepared in duplicates. The band immediately below the Cdc25A band represents a nonspecific species. Treatment with lower concentrations of camptothecin (100–300 nM) overnight also conferred Cdc25A degradation (data not shown). B, doxorubicin induces Cdc25A degradation in H1299 cells. H1299 cells were treated with or without 500 nM doxorubicin in duplicate for 24 h. Protein samples were analyzed for Cdc25A, Chk1, P-Chk1, actin, and Cdc25C profiles.

**Fig. 2.** Chk1 directly mediates the DNA damage-induced proteolysis of Cdc25A. A, Chk1 mediates endogenous Cdc25A degradation in response to camptothecin. H1299 cells were transfected with Chk1 scr- or siRNA. Cells were then treated with 3 μM camptothecin for 0, 3, and 6 h and harvested for Western analysis. Cdc25A, Chk1, and actin levels were determined by immunoblots. B, Chk1 mediates degradation of ectopically expressed Cdc25A. H1299 cells were transfected with 0.1 μg of Cdc25A plasmid, along with Chk1 scr- or siRNA. Cells were then treated with 3 μM camptothecin for 0, 3, and 6 h and harvested for Western analysis. Cdc25A, Chk1, and actin levels were determined by immunoblots. C, Chk1 mediates Cdc25A degradation in response to doxorubicin. H1299 cells were transfected with Chk1 scr- or siRNA. Cells were then treated with 500 nM doxorubicin for 24 h and harvested for Western analysis. Cdc25A, Cdc25C, Chk1, and actin levels were determined by immunoblots.
in doxorubicin-treated Chk1-deficient cells (14% in profile 4 versus 1% in profile 2), suggesting that abrogation of the checkpoint by Chk1 knockdown induced extensive cell death.

In contrast to its role in the G2 checkpoint, the involvement of Chk1 in S phase arrest is not as widely accepted. To convincingly demonstrate the role of Chk1 in S checkpoint, we carried out a BrdUrd labeling experiment with camptothecin-treated H1299 cells to better characterize the expected S phase arrest. First, in the absence of camptothecin, there were no differences in the labeling profiles among mock transfected cells, cells transfected with Chk1 siRNA, and cells transfected with scrRNA (Fig. 3B, upper panels). With camptothecin treatment, we noticed a dramatic decrease in BrdUrd labeling of the S phase cells in either mock or scrRNA-transfected cells (33 to 1, and 28 to 1%, respectively), consistent with the drug-induced S phase arrest. However, Chk1 siRNA transfection effectively maintained the DNA synthesis rate (27 to 21%) even in the presence of DNA damage, demonstrating abrogation of the arrest and further indicating that Chk1 also mediates camptothecin-induced S checkpoint. Chk1 immunoblot was performed to confirm the elimination of Chk1 protein by the siRNA (data not shown).

The Function of Cdc25A in G2/M Transition—So far, we have shown the Chk1 mediates Cdc25A degradation and arrests cells in either S or G2/M when induced by DNA-damaging agents. To further confirm that Cdc25A plays a role in G2/M transition in addition to its more widely accepted function in G1/S progression, we studied the cell cycle profiles of H1299 cells transiently transfected with increasing amounts of expression plasmids encoding either Cdc25A or Cdc25C (Fig. 4A). The cells were treated with doxorubicin to induce G2 arrest; FACS analysis was performed to determine whether Cdc25 overexpression could abrogate the DNA damage-induced G2 arrest. Control cells in the absence of doxorubicin showed a normal predominant distribution in G1 phase and a relatively minor G2/M population (right profile, panel 1). In contrast, doxorubicin-treated cells exhibited a large increase of the G2/M peak, indicating G2 arrest (left profile, panel 1). With Cdc25C overexpression (panel 2), we observed a dose-dependent abrogation of the G2 arrest starting at the second dose (100 ng/well transfection), consistent with its published role in promoting G2/M phase progression through dephosphorylation of Cdc2 (7). In comparison, Cdc25A overexpression (panel 3) also conferred a similar abrogation of the G2 arrest in a dose-dependent manner starting with the second dose (100 ng/well transfection), indicating that Cdc25A mediates G2/M transition with similar efficacy as Cdc25C. As a control, transfection of cells with increasing amounts of empty vector did not confer any abrogation (data not shown).
To confirm the overexpression of Cdc25A in the above experiment and further investigate the underlying mechanism of the observed abrogation of G2 arrest, we subjected the same cell samples to immunoblot analysis for Cdc25A and Cdc2/Cdk1 (Fig. 4B). Because Cdc2 is the kinase directly responsible for G2/M transition, and its dephosphorylation at tyrosine 15 by Cdc25C has been shown to cause its activation and M phase progression (6), we speculated that it might also be the downstream target of Cdc25A. Transfection of 50 ng of the Cdc25A plasmid generated a weak Cdc25A signal, which disappeared after doxorubicin treatment (Fig. 4B, lanes 3 and 4), indicating Chk1-mediated degradation in response to DNA damage. In contrast, transfection with 200 and 500 ng of the plasmid conferred significantly higher amounts of Cdc25A, and more importantly, the overexpressed Cdc25A became mostly resistant to drug-induced proteolysis (lanes 5–8). Correspondingly, 200 and 500 ng but not the 50-ng transfection of Cdc25A efficiently decreased the doxorubicin-induced phosphorylation of Cdc2 at tyrosine 15 (Cdc2 Y15P), suggesting that Cdc25A activates Cdc2 through direct dephosphorylation of Cdc2 Y15P. Total Cdc2 protein levels did not show significant variation among the different samples (data not shown). The 500-ng transfection conferred higher Cdc25A accumulation than the 200-ng transfection, resulting in more complete elimination of Cdc2 Y15P signal (compare lanes 6 and 8), in line with its more complete abrogation of the G2 arrest (Fig. 4A). As a negative control, transfection of 500 ng of a plasmid expressing Cdc25A C430S, a phosphatase-dead inactive mutant (15), also resulted
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in doxorubicin-resistant accumulation of Cdc25A to a level that is even higher than transfection with same amount of wild type construct (compare lanes 9 and 10 and 7 and 8). However, this mutant Cdc25A is unable to dephosphorylate Cdc2 and decrease the Cdc2 Y15P signal, thus confirming that the phosphatase activity of Cdc25A is required for Cdc2 activation. Cdc2 total protein level was also studied for this experiment and found to be mostly constant among the different samples (data not shown).

Fig. 4C shows Cdc25C overexpression-induced dephosphorylation of Cdc2. Similar to Cdc25A, Cdc25C overexpression also led to a decrease in the Cdc2 Y15P signal in a dose-dependent manner, suggesting that Cdc25C abrogates DNA damage-induced G₂/M arrest via re-activation of Cdc2 through its dephosphorylation. This corroborates previous reports showing that Cdc2 is a direct downstream target of Cdc25C.

**DISCUSSION**

Chk1 was proposed to play a role in the S or G₂ checkpoints induced by various DNA-damaging agents including UV, IR, and topoisomerase I inhibitors (1, 4, 8, 22). This is supported by the observation that in normal human fibroblasts (MJ90) and also in fibroblasts derived from patients with ataxia telangiectasia (AT), Chk1 is expressed specifically at the S to M phase of the cell cycle at both the RNA and protein levels and its activity is readily detected at the S to M phase of the cell cycle (23). Nonetheless, the direct involvement of Chk1 in these processes have not been convincingly demonstrated and the underlying mechanisms for these checkpoints still remain controversial. We report here that Cdc25A protein undergoes Chk1-mediated degradation in response to various DNA-damaging drugs, and this degradation coincides with and determines the resultant S or G₂ checkpoints. Therefore, Chk1 elimination through siRNA leads to abrogation of both Cdc25A proteolysis and the S or G₂ checkpoints.

During the final preparation stage of this article, a new report appeared showing that Cdc25A is subject to Chk1-mediated proteolysis in response to double-stranded DNA breaks generated by IR (24). This contradicts a previous study that demonstrated that Chk2, not Chk1, is primarily responsible for mediating IR-induced Cdc25A degradation (25). In this study, we focused on two major topoisomerase inhibitors (camptothecin and doxorubicin), the mainstay of cancer chemotherapy. Camptothecin is the most commonly used first-line and second-line treatment for colon cancer, whereas doxorubicin is widely used to combat breast, stomach, and cervical cancers (26). Because IR and topoisomerase inhibitors confer DNA damage through different mechanisms (see below), they may activate distinct signal transduction pathways. The mechanism of cell cycle arrest by the topoisomerase inhibitors has not been clearly established. This lack of theoretical guidance has seriously impeded the improvement of current chemotherapy and rational design of next generation chemotherapeutics. Our study directly addressed this urgent issue by demonstrating that Cdc25A and Cdc25C may be both required for G₂/M progression. When cells enter mitosis, there is a dramatic increase of cyclin B-Cdc2 kinase activity, which is required for early stage mitotic progression (29). To maintain this requisite level of Cdc2 kinase activity, a sustained pool of Cdc25 phosphatase activity may be necessary. As mentioned previously, among the three Cdc25 members, Cdc25B is regarded as an “initiator” phosphatase that is primarily responsible for creating the first stimulation of cyclin B-Cdc2 kinase activity to set in motion the mitotic process (11, 12). However, Cdc25B is an unstable protein with a short half-life (30), and it is degraded in a proteasome-dependent manner upon M phase entry (31). Thus the burden of maintaining the active cyclin B-Cdc2 kinase falls to the other members of the family, namely Cdc25A and Cdc25C. We hypothesize that the endogenous level of each protein is insufficient to sustain the necessary threshold of Cdc2 kinase activity, thus the presence of both are required for proper M phase progression. As shown in Fig. 4, this dual requirement can be clearly circumvented by the transient overexpression of either Cdc25A or Cdc25C.

As discussed above, Cdc25C knockout cells show little mitotic abnormalities (28). This does not mean that Cdc25C normally plays no role in G₂/M progression. It only means that Cdc25C is not essential for this process. Apparently other members of the Cdc25 family are able to compensate for its loss. We speculate that in Cdc25C-null cells, Cdc25A may undergo certain kinds of up-regulation, either at the mRNA or protein levels, or at the phosphatase activity level through post-translational modifications, to still maintain a normal pool of Cdc25 phosphatase activity.

**Will Different DNA-damaging Agents, Especially Topoisomerase Inhibitors, All Induce Cdc25A Degradation?**—Previously it was reported that hydroxyurea, an antimetabolite-inducing S phase arrest, also stimulated Cdc25A degradation (32). We showed here that camptothecin, a topoisomerase I poison conferring S arrest, induces the same process specifically through Chk1. It is not clear what distinguishes these two
phenotypically different forms of S checkpoint. It will be interesting to know whether the DNA polymerase inhibitor aphidicolin, causing S phase arrest through yet another mechanism, also leads to Cdc25A proteolysis.

IR generates DNA damage through creating double-stranded DNA breaks, this process does not directly involve topoisomerase. In contrast, camptothecin and doxorubicin induce DNA damage through inhibition of topoisomerase I and II, respectively. Topoisomerases regulate DNA architecture by sustaining the correct DNA superhelicity and resolving intertwined DNA strands, particularly during DNA replication. Topoisomerase I generates single-stranded breaks, whereas topoisomerase II creates double-stranded breaks. The cytotoxicity of their inhibitors generally stems from the stabilization of topoisomerase-DNA covalent cleavage intermediates (also known as cleavage complexes). Besides camptothecin and doxorubicin, other clinically effective drugs acting in this manner include topotecan and SN-38 (topoisomerase I) as well as mitoxantrone and etoposide (topoisomerase II) (26). It will be interesting to ascertain whether we can extend our current findings to the other topoisomerase poisons. If yes, this will further broaden the utility of Chk1 inhibitors in the potentiation of cancer chemotherapeutics.

In conclusion, our results showed that the Chk1/Cdc25A pathway mediates both S and G2 checkpoints. This finding defines Chk1 as a more ubiquitous cell cycle checkpoint mediator and hence significantly broadens the application of Chk1 inhibitor in the potentiation of various cancer chemotherapeutics.

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