ATR-dependent Activation of p38 MAP Kinase Is Responsible for Apoptotic Cell Death in Cells Depleted of Cdc7*

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Cdc7 is a serine/threonine kinase that plays essential roles in the initiation of eukaryotic DNA replication and checkpoint response. In previous studies, depletion of Cdc7 by small interfering RNA was shown to induce an abortive S phase that led to the cell cycle arrest in normal human fibroblasts and apoptotic cell death in various cancer cells. Here we report that stress-activated p38 MAP kinase was activated and responsible for apoptotic cell death in Cdc7-depleted HeLa cells. The activation of p38 MAP kinase in the Cdc7-depleted cells was shown to depend on ATR, a major sensor kinase for checkpoint or DNA damage responses. Only the p38 MAP kinase, and not the other stress-activated kinases such as JNK or ERK, was activated, and both caspase 8 and caspase 9 were activated for the induction of apoptosis. Activation of apoptosis in Cdc7-depleted cells was completely abolished in cells treated with small interfering RNA or an inhibitor of the p38 MAP kinase, suggesting that p38 MAP kinase activation was responsible for apoptotic cell death. Taken together, we suggest that the ATR-dependent activation of the p38 MAP kinase is a major signaling pathway that induces apoptotic cell death after depletion of Cdc7 in cancer cells.

Cdc7 is an evolutionally conserved serine/threonine kinase that is essential for the initiation of eukaryotic DNA replication (1, 2). Cdc7 kinase activity is required for the activation of individual replication origins throughout S phase presumably by phosphorylating components of the pre-replicative complex (pre-RC) such as the Mcm proteins. Although the Cdc7 protein level is constant during the cell cycle, its kinase activity fluctuates during this period because of its binding to the regulatory subunit, Dbf4, whose level peaks at S phase (3, 4). Whereas only one regulatory subunit has been identified in yeast systems, mammals and *Xenopus* contain an additional regulatory subunit, Drf1 (5). Both Dbf4 and Drf1 appear to be important for DNA replication (3, 5).

There are several reports suggesting that Cdc7 kinase also plays an important role in the checkpoint response to DNA damage or replication stress. The Cdc7 kinase complex appears to be a direct target of Rad53/Cds1, yeast homologues of mammalian Chk2 kinase, which inhibits DNA replication during checkpoint responses (6, 7). Single-stranded DNA, which is generated by various genotoxic stresses, also targets the Cdc7/Dbf4 kinase via the activation of ATR, resulting in the inhibition of DNA synthesis in the *Xenopus* system (8). In mammalian cells, contradictory reports suggest that the Cdc7 kinase is either a target of the Chk1 pathway or an activator of the Chk1 kinase (9, 10).

When cells are subjected to various kinds of genotoxic stress, such as DNA damage or inhibition of DNA replication, complex checkpoint pathways are activated that arrest the cell cycle and facilitate DNA repair. ATM-Chk2 and ATR-Chk1 are major factors that control checkpoint responses in mammalian cells. ATM is a protein kinase that is activated primarily by double-strand breaks (DSBs), whereas ATR is a chromatin-binding protein kinase that is activated by single-stranded DNA generated by factors that block replication and/or lead to the collapse of replication forks. The ATM/ATR kinases phosphorylate and activate Chk1/Chk2 kinases, which, in turn, activate various proteins involved in cell cycle control, DNA repair, and cell survival (2, 11).

The tumor suppressor protein, p53 is a major downstream effector of these kinase pathways (11, 13, 14). In normal cells the activation of p53 results in cell cycle arrest primarily by up-regulating the expression of p21. However, when DNA damage is extensive, p53 induces cell death by activating intrinsic or extrinsic apoptotic pathways (15).

Another important player in the cellular response to genotoxic stress is the p38 MAP kinase (p38 MAPK).2 Originally, p38 MAPK was identified as a stress-activated protein kinase that responded to various cellular stimuli including cytokines, UV irradiation, heat/osmotic shock, and oxidative stress (16). The activity of p38 MAPK was reported to play an important role in G2/M checkpoint response after UV irradiation (17), and MAPK-activated protein kinase-2 (MK2) appears to be the downstream effector kinase of p38 MAPK for the checkpoint response (18). The sustained activation of p38 MAPK and c-Jun N-terminal kinase (JNK) is also responsible for cisplatin (cis-diaminedichloro-platinum II)-induced apoptosis by activating the death receptor pathway for apoptosis (19, 20).

Recently, the down-regulation of Cdc7 kinase activity in mammalian cells was reported to induce apoptotic cell death in

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2 The abbreviations used are: MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; pre-RC, pre-replicative complex; DSBs, double-strand breaks; MK2, MAPK-activated protein kinase 2; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; cisplatin, cis-diaminedichloro-platinum II; PARP, poly(ADP-ribose) polymerase.

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various cell lines. The inactivation of Cdc7 kinase in mouse ES cells resulted in cell cycle arrest at the S phase, and eventually induced p53-dependent cell death (21). The depletion of Cdc7 kinase by transfecting small interfering (si) RNAs caused cell cycle arrest at S phase in normal human fibroblast cells, but led to the apoptotic cell death in various cancer cell lines (22). Because normal and cancer cells show different responses following depletion of Cdc7 kinase, Cdc7 appears to be an attractive target for cancer drug therapies. In normal human fibroblast cells, the tumor suppressor protein, p53 appears to play an important role leading to cell cycle arrest after Cdc7 depletion (22). However, the mechanisms or pathways that induce apoptosis in Cdc7 depleted cancer cells are currently unknown.

In this study, we examined the checkpoint and apoptotic pathways that were affected by the depletion of Cdc7 kinase in HeLa cells. Our findings indicate that the depletion of Cdc7 activated the p38 MAPK in an ATR-dependent manner and led to the apoptotic cell death of HeLa cells. Based on these results, we propose that the ATR-dependent activation of p38 MAPK is a major signaling pathway that induces apoptotic cell death after replication stress in cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—HeLa (human cervical adenocarcinoma), U2OS (human osteosarcoma), and HT-29 (human colon adenocarcinoma) cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum and antibiotics. HCT116 (human colon cancer) p53+/+ or p53−/− and SNU-C2A (human colorectal adenocarcinoma) were cultured in RPMI medium, supplemented with 10% fetal bovine serum and antibiotics. All cell culture reagents were purchased from Welgene Inc (Seoul, Korea). Cells were maintained at 37 °C in a 5% CO2 humidified incubator. The reagents staurosporine, caffeine, cisplatin, nocodazole, and SB202190 were purchased from Sigma-Aldrich.

**Analysis of Cell Cycle and Apoptosis**—For cell cycle analysis, cells were transfected with siRNAs and further incubated for 48–72 h. Cells were collected after treatment with trypsin, washed with phosphate-buffered saline, and fixed with 70% ethanol in phosphate-buffered saline. The cells, resuspended in phosphate-buffered saline containing RNase A (2 µg/ml), were incubated at 37 °C for 30 min. After incubation with 100 µg/ml propidium iodide for 10 min at 37 °C, samples were analyzed with a FACScalibur flow cytometer (Becton Dickinson). Analysis of apoptosis was carried out with the Annexin V-FITC apoptosis detection kit (Calbiochem) according to the manufacturer’s instructions.

**Preparation and Transfection of siRNAs**—The sense strand sequences of individual siRNAs used in this study were as follows: CDC7, 5′-AAGCUACAGGAAAGGUGTT-3′; CDC7 control, 5′-AAGCUAGAAAGAAGUGTT-3′; ATR, 5′-CUCCGGUGUAGUUGCUUA-3′; p38-α, 5′-AAGGC-CUACCUCCUGGU-3′, and MK2, 5′-UGACCAU-CACCGAUAU-3′. All chemically synthesized siRNAs were from Samchully Pharm (Daejon, Korea). Transfections of siRNAs (120 nM) were performed with Oligofectamine (Invitrogen) following the manufacturer’s instructions.

**Immunoblotting**—For immunoblotting, proteins were extracted with lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 1 mM benzamidine, and 5 µg/ml pepstatin A. Approximately 30 µg of protein from each lysate was subjected to SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined by Bradford assays (Bio-Rad). The antibodies against phospho-ATM (Ser-1981), phospho-p38 (Thr-180/Tyr-182), phospho-Chk1 (Ser-317), phospho-Chk2 (Thr-68), phospho-MK2 (Thr-334), phospho-JNK (Thr-183/Tyr-185), phospho-ERK (Thr-202/Tyr-204), phospho-Rb (Ser-807/811), phospho-MKK3/6 (Ser-189/207), phosphor-MKK4 (Ser-257/Thr-261), p38, cleaved caspase3, cleaved caspase 8, cleaved caspase 9, PARP, JNK, and cyclin A were purchased from Cell Signaling Technology. Antibodies against Cdc7, ATR, Chk1, and Chk2 were purchased from Abcam, and antibodies against actin, cyclin E, Cdc25A, and Rb were purchased from Santa Cruz Biotechnology. The antibody against cyclin B1 was purchased from Calbiochem.

**RESULTS**

**Cdc7 Depletion Does Not Elicit the Canonical Replication or DNA Damage Checkpoint Pathways, but Leads to the Apoptotic Cell Death of HeLa Cells**—To investigate the cellular responses to the depletion of Cdc7 kinase, we repressed the expression of Cdc7 in HeLa cells using siRNA. SiRNA transfection efficiently reduced the expression of the Cdc7 kinase. As shown in Fig. 1A, the level of remaining Cdc7 was below detection 2 days following transfection. Because Cdc7 plays an essential role in the initiation of DNA replication, we expected Cdc7-depleted cells to be arrested at the G1/S or S phase of the cell cycle. However, flow cytometry analysis did not show significant accumulation of G1 or S phase cells after Cdc7 depletion but instead revealed a significant increase of sub-G1 cells (20% in this particular experiment) (Fig. 1A). The products of apoptosis, such as the cleaved forms of caspase 3 and poly(ADP-ribose) polymerase (PARP), were detected by Western blot analysis (Fig. 1A, right panel). Induction of apoptosis was also confirmed by annexin V staining of Cdc7-depleted cells (Fig. 1B). In general, these cells had an abnormal morphology and most eventually died after postincubation for 3–4 days (data not presented).

To precisely examine the effect of Cdc7 depletion on cell cycle progression, we synchronized the Cdc7-depleted cells at the G2/M phase with nocodazole, and then analyzed the progression of the cell cycle and induction of apoptosis after removal of nocodazole (Fig. 1C). Although there was some delay in the progression of the cell cycle from G2/M to G1 in Cdc7-depleted cells, G1 cells started to appear 6 h after removal of nocodazole, and a significant subset of cells (32%) were in the G1 phase after 12 h. These findings suggested that progression of the cell cycle from G2/M to G1 was not defective in the Cdc7-depleted cells. When cells were incubated further, however, cells containing 4N DNA did not re-appear, while the number of cells in the G1 phase decreased and those in the S phase increased. Instead of 4N DNA cells, there was a significant increase of sub-G1 cells, suggesting that cells entering the S phase in the absence of Cdc7 rapidly suffered apoptotic cell death.
A Stress-activated Protein Kinase, p38 MAPK, Is Activated by Depletion of Cdc7 in HeLa Cells—We examined whether the depletion of Cdc7 induced apoptosis in various cancer cell lines. For this purpose, the activation of caspase 3 and PARP (Fig. 2A), as well as the generation of a sub-G1 cell populations (Fig. 2B), were examined after Cdc7 depletion in several p53-positive (HCT 116, U2OS, HT-29, SNU-C2A) and p53-negative (HCT116 E6) cancer cells. Apoptosis was induced in all tested cell lines after Cdc7 depletion although some differences in the level of activation were noted. These results suggest that apoptotic cell death is a general response found in most cancer cells after depletion of Cdc7. We also concluded that the loss of p53 was not essential for this effect because there was no correlation between the status of p53 and the level of apoptosis.

We then searched for the pathway that was responsible for the induction of apoptosis in Cdc7-depleted cancer cells. Although the tumor suppressor, p53, generally plays an important role in the induction of apoptosis in response to genotoxic stress, the data shown in Fig. 2 suggest that this protein is not involved in the apoptotic cell death of Cdc7-depleted cancer cells. Another possible candidate is p38 MAPK, which has been shown to play roles in the checkpoint response and apoptosis in response to a variety of genotoxic stresses (16). Indeed, the activation of p38 MAPK was detected after Cdc7 depletion in HeLa cells (Fig. 3A). Cdc7 depletion resulted in the increased phosphorylation of p38 MAPK, which activated p38 MAPK, leading to the elevated phosphorylation of MK2, a major downstream target of p38 MAPK, which has been shown to play roles in the checkpoint response and apoptosis in Cdc7-depleted cancer cells. Although some differences in the level of apoptosis were noted, these results suggest that apoptotic cell death is a general response that can be observed in most cancer cells after depletion of Cdc7.

Because p38 MAPK is a family member of the stress-activated protein kinases, which are activated under a variety of stress conditions, we examined whether other MAP kinase members were also affected by the depletion of Cdc7 kinase. Treatment of cells with a cross-linking agent, cisplatin, increased the phosphorylation of all stress-activated protein kinases, which are activated under a variety of genotoxic conditions (16). Indeed, the activation of p38 MAPK was detected after Cdc7 depletion in HeLa cells (Fig. 3A). Cdc7 depletion resulted in the increased phosphorylation of p38 MAPK, which activated p38 MAPK, leading to the elevated phosphorylation of MK2, a major downstream target of p38 MAPK in the checkpoint response.

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tion, we searched for upstream effectors of the p38 MAPK. In most cases, cellular responses to DNA damage or replication stress are initiated by the activation of sensor kinases, such as ATM or ATR. Therefore, we examined whether these sensor kinases were involved in the activation of p38 MAPK. When Cdc7-depleted cells were treated with caffeine, a well known inhibitor of the ATM and ATR kinases, the activating phosphorylation of p38 MAPK and MK2 was completely blocked (Fig. 4).

Previous results have indicated that ATM is not activated in Cdc7-depleted cells (Fig. 1D), suggesting that the ATR sensor kinase was responsible for the activation of p38 MAPK. Indeed, the siRNA-targeted down-regulation of ATR alone completely eliminated the activation of both p38 MAPK and MK2 (Fig. 4B). Thus, the action of ATR is required for the activation of p38 MAPK in Cdc7-depleted cells. Generally, p38 MAPK is known to be activated by the upstream MAPK kinases (MAPKKs), such as MKK3 and MKK6. However, we did not observe a clear increase in the activating phosphorylation of MKK3/MKK6 or MKK4 after Cdc7 depletion (Fig. 4C).

To examine the role of p38 MAPK on apoptosis in Cdc7-depleted cells, we tested whether the induction of apoptosis was prevented by inhibiting the p38 pathway. Treatment of cells with SB202190, a specific inhibitor of p38 MAPK, completely prevented cleavage of caspase 3 and PARP (Fig. 5A). Further- more, the down-regulation of p38 MAPK with siRNAs against p38α also inhibited the activation of apoptosis in Cdc7-depleted cells (Fig. 5B). In contrast, depletion of MK2 did not prevent the activation of the apoptotic pathway (Fig. 5C). These results indicate that the activation of p38 MAPK is responsible for inducing apoptosis in Cdc7-depleted cells, and MK2 is not a downstream effector in this process. When the pathways for apoptotic activation was examined, cells depleted of Cdc7 showed an increase of FAS-L and the activation of caspase 8 as well as the activation of caspase 9 (Fig. 6). Therefore, the activation of p38 MAPK appears to induce apoptotic cell death by
DISCUSSION

In this study, we demonstrated that the p38 MAPK, a stress-activated protein kinase, is an essential factor required for apoptotic cell death in Cdc7-depleted cancer cells. Among three well known stress-activated protein kinases, JNK, ERK, and p38 MAPK, p38 MAPK is the only one activated in Cdc7-depleted cells, and this activation requires ATR, a major sensor kinase for checkpoint response. The activation of p38 MAPK leads to the apoptotic cell death in Cdc7-depleted cells by activating both death receptor and mitochondrial apoptosis pathways.

As shown in this study, the cellular apoptotic pathway that is activated by Cdc7 depletion appears to be similar to that activated by treatment with cisplatin. Cisplatin treatment results in long-term activation of p38 MAPK, which can persist for several days. This long-lasting activation of p38 MAPK, which is accompanied by the sustained up-regulation of AP-1 and FAS-L, is required for the induction of apoptosis (19). Apoptosis occurs late and typically starts 2–4 days following exposure to cisplatin (23). In contrast, the induction of apoptosis in Cdc7-depleted cells appeared rapidly within a few hours after entry into the S phase (Fig. 1C). We reason that this difference may be due to the defects in the Chk1/Chk2 pathway in Cdc7-depleted cells (Fig. 1D). Cells treated with cisplatin activate both the Chk1/Chk2-dependent checkpoint pathway as well as p38 MAPK. It has been reported that inactivation of the Chk1/Chk2-dependent checkpoint pathway increased apoptotic cell death induced by cisplatin or ionizing radiation (24, 25). Therefore, we suggest that cells depleted of Cdc7 are more susceptible to apoptotic cell death because of defects in Chk1/Chk2 pathways.

Generally, when cells undergo replication stress caused by DNA damages or the presence of inhibitors of replication, the Chk1/Chk2-dependent checkpoint pathways are activated to prevent cell cycle progression and DNA synthesis (26, 27). On the other hand, Cdc7 depletion did not elicit activation of the Chk1/Chk2-dependent checkpoint pathways. Because Cdc7 is essential for the initiation of DNA replication, the lack of checkpoint activation in Cdc7-depleted cells may reflect an insufficient accumulation of single stranded DNA regions or formation of double strand breaks, both of which are recognized by the sensor proteins ATR and ATM, respectively. However, the activation of ATR in Cdc7-depleted cells appeared to be normal since the activation of p38 MAPK requires the activity of ATR. These results indicate that Cdc7 is not only required for the initiation of DNA replication, but is also required for the ATR-dependent activation of Chk1/Chk2. Consistent with these results, it has recently been suggested that Cdc7 kinase mediates the phosphorylation of Claspin, which is required for replication checkpoint (10). As a result of these two functions, Cdc7 depletion leads to apoptotic cell death, which would not occur when other replication proteins are inactivated.

Among the members of the p38 MAPK family (p38α, β, γ, and δ), p38α is the most abundant kinase expressed in most cell types (28–30). p38α is also known to sensitize cells to apoptosis by both up-regulation of pro-apoptotic proteins and down-regulation of survival pathways (31). In the case of cells depleted of Cdc7, the induction of apoptosis also appears to occur via the activation of p38α, because the siRNA against p38α almost completely prevented the activation of caspase 3 (Fig. 5B).

In this study, the activation of p38 MAPK in Cdc7-depleted cells was shown to depend on ATR, a major sensor protein...
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required for checkpoint responses (Fig. 5). In contrast, the activation of p38 MAPK in UV-irradiated cells did not require ATM or ATR (17). These differences suggest that the mechanism of p38 MAPK activation varies depending on the stress conditions. Although the mechanism by which ATR activates p38 MAPK remains to be resolved, it is plausible that ATR affects the activities of the MAPKKs or MAPK kinase kinases directly or indirectly. MKK3 and MKK6 are generally known to activate p38 MAPK, and other MAPKKs, such as MKK4, may also contribute to the activation of p38 MAPK as was shown in UV-irradiated cells (32). To date, however, we have not detected a clear activation of MKK3, MKK6, and MKK4 in Cdc7-depleted cells (Fig. 4C). Despite this, it is still possible that several MAPKKs are involved in the activation of p38 MAPK in Cdc7-depleted cells.

Although the apoptosis-inducing activity of p38 MAPK has been well established in many systems, p38 MAPK activation also plays important roles in checkpoint response to DNA damages. One of the p38 MAPK targets for checkpoint responses is MK2, a kinase that plays a similar role to Chk2. It was reported that p38-dependent activation of MK2 led to the phosphorylation of Cdc25B or degradation of Cdc25A, which was required for the UV or cisplatin-induced checkpoint pathway (18, 33). The role of the p38 MAPK/MK2 pathway in cell survival was further confirmed by demonstrating that the depletion of MK2 after DNA damage reduced cell survival in p53-deficient cells (33). Although p38-dependent activation of MK2 was observed in Cdc7-depleted cells (Fig. 3A), a majority of the cells still underwent apoptotic cell death instead of cell cycle arrest. One possible explanation for this result is the absence of the Chk1/Chk2 pathway in Cdc7-depleted cells. Exposure of cells to UV or other genotoxic stress activated the MK2 as well as the Chk1/Chk2 pathways. The combined action of these two pathways appeared to be important for cell cycle arrest (18, 33). The activation of the MK2 pathway alone in the absence of the Chk1/Chk2 activation, which occurred in Cdc7-depleted cells, may not be sufficient to arrest the cell cycle.

As previously reported, Cdc7 depletion did not elicit apoptotic cell death in normal human fibroblast cells (22), but the activation of p38 MAPK was still observed (data not presented). However, the activation of p38 MAPK in normal cells did not induce apoptosis. In normal cells, there are many redundant checkpoint pathways leading to cell cycle arrest (Fig. 7). Although the Chk1/Chk2 pathway is rendered defective in Cdc7-depleted normal cells, the activation of p53 and the concomitant up-regulation of p21, an inhibitor of cyclin-dependent kinases can lead to cell cycle arrest at the G1/S boundary (34, 35). p38-dependent activation of MK2 also plays a significant role in cell cycle arrest and cell survival (18, 36). However, the loss of normal checkpoint functions and the up-regulation of signaling pathways that lead to rapid cell proliferation are common in many different cancer cells (11, 12). The lack of p53 and Chk1/Chk2 pathways in the presence of a replication stress (as observed in Cdc7-depleted HeLa cells) may further amplify the signals for ATR/p38 MAPK activation, which would eventually lead to apoptotic cell death (Fig. 7). Aberrations in the cell cycle or checkpoint related proteins, such as Rb, p16, cyclins, and Cdc25, would also affect the sensitivity of cancer cells to the depletion of Cdc7. Based on these observations Cdc7 appears to be an attractive target for the development of anticancer drugs. Furthermore, drugs that specifically inhibit Cdc7 kinase activity would be particularly effective in the treatment of cancer cells that harbor defects in the checkpoint function such as p53.

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