Checkpoint Kinase 1 Is Cleaved in a Caspase-Dependent Pathway during Genotoxic Stress-Induced Apoptosis

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Checkpoint kinase 1 (Chk1) plays important roles in genotoxic stress-induced cell cycle checkpoint and in normal cell cycle progression. Here, we show that Chk1 is cleaved in the treatment of apoptotic dose of etoposide (ETP) or cisplatin (CIS) but not of viable dose in HeLa S3 cells. The cleavage of Chk1 was completely inhibited by an irreversible and cell-permeable pan-caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD-fmk). These results identify Chk1 as a novel substrate that is cleaved by a caspase-dependent manner during genotoxic stress-induced apoptosis. Our data may also indicate the existence of a novel Chk1-regulated apoptotic pathway.

Key words checkpoint kinase 1 (Chk1); genotoxic stress; cell cycle checkpoint; DNA repair; apoptosis; caspase

Chk1 is evolutionally conserved a serine/threonine protein kinase that guarantees genomic integrity through checkpoint activation after DNA damage.[1)] In response to various genotoxic stress, Chk1 is phosphorylated and activated in Ataxia-telangiectasia mutated and Rad-3 related kinase (ATR)- or Ataxia telangiectasia mutated kinase (ATM)-dependent pathway.[2)] Subsequently, the activated Chk1 phosphorylates various downstream effectors, such as CDC25 phosphatases,[3,4)] p53,[5,6)] and p73[7,8)] to abort cell cycle progression. This cell cycle arrest is considered to allow time for cells to decide repair or apoptosis as to the levels of DNA damage.

So far, ATR-Chk1 signaling pathway has been characterized well.[1)] In response to genotoxic stress, ATR-ATRIP complex and Claspin, a protein of high binding affinity for branched DNA, collaborate in the activation of Chk1.[9)] *Chk1* disruption in mice causes embryonic lethality[10—12)] as in the case of *ATR* disruption.[13,14)] Chk1-downregulated by RNAi or -disrupted by knock-out mammalian cells become to be hypersensitive against various genotoxic stress.[10—12,15)] Recently, it has been reported that Chk1 is phosphorylated in a genotoxic stress-independent manner[16)] and required for mitotic progression.[17)] Thus, the ATR-Chk1 signaling pathway is essential not only in genotoxic stress responses but also in embryonic development and normal cell cycle.

In genotoxic stress-induced apoptosis, it is suitable and effective for apoptotic commitment and execution that DNA repair machinery is inactivated by caspases. In fact, various DNA repair factors including poly (ADP-ribose) polymerase-1 (PARP-1), DNA-dependent protein kinase (DNA-PK) and ATM are cleaved by caspases and inactivated.[18—22)] However, although Chk1 is an important factor that regulates genotoxic stress-induced cell cycle checkpoints, the direct involvement in apoptosis has not yet been reported. In this study, we first demonstrate that Chk1 is cleaved in response to apoptotic DNA damage, but not to viable damage. We also show that an irreversible and cell-permeable pan-caspase inhibitor, z-VAD-fmk,[22—24)] completely blocks Chk1 cleavage in ETP-induced apoptosis. These findings indicate that the caspase-mediated cleavage of Chk1 may be a novel apoptotic regulatory pathway.

MATERIALS AND METHODS

Cell Culture and Drug Treatments HeLa S3 cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin G, and 100 U/ml streptomycin and maintained 0.1—1$\times10^5$ cells/cm^2 at cell density. CIS, ETP, and z-VAD-fmk were purchased from WAKO and Peptide Institute, respectively. Exponentially growing cells were seeded at a cell density of 1—2$\times10^4$ cells/cm^2 and incubated for overnight. In ETP or CIS treatment, the viable and apoptotic doses in drug treatments were determined as follows: the viable dose; a maximum dose, which is detected no band of cleaved PARP-1 by Western blotting and no subG1 peak by flowcytometric analysis using propidium iodide staining: the apoptotic dose; a minimum dose, which is detected a maximum cleaved PARP-1 (data not shown).

Antibodies and Western Blotting Mouse anti-Chk1 monoclonal antibody (clone MCS-310) and goat anti-Chk1 polyclonal antibody were purchased from MBL and R&D Systems, respectively. Rabbit anti-Chk1 polyclonal antibody and rabbit anti-Chk1 (pSer317) polyclonal antibody were obtained from Cell Signaling Technology. Mouse anti-β-actin monoclonal antibody (clone AC-15) and mouse anti-PARP-1 monoclonal antibody (clone C2-10) were from SIGMA and WAKO, respectively. Goat anti-mouse IgG HRP-conjugate, goat anti-rabbit IgG HRP-conjugate, and donkey anti-goat IgG HRP-conjugate were from Jackson Immunological Research.

Cell extracts were prepared in 1$\times$SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, and 0.1% bromophenol blue) and boiled for 5 min. Proteins in the extracts were separated by 8% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 2.5% skim milk and 0.25% BSA in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 h at room temperature, and then probed with appropriate primary antibodies for overnight at 4 °C or for 2 h at room temperature. After the membranes were washed with TTBS, incubated with the appropriate secondary antibody for 1 h at room temperature. After washing the membrane with TTBS, the blotted proteins were visualized with Light Capture Sys-

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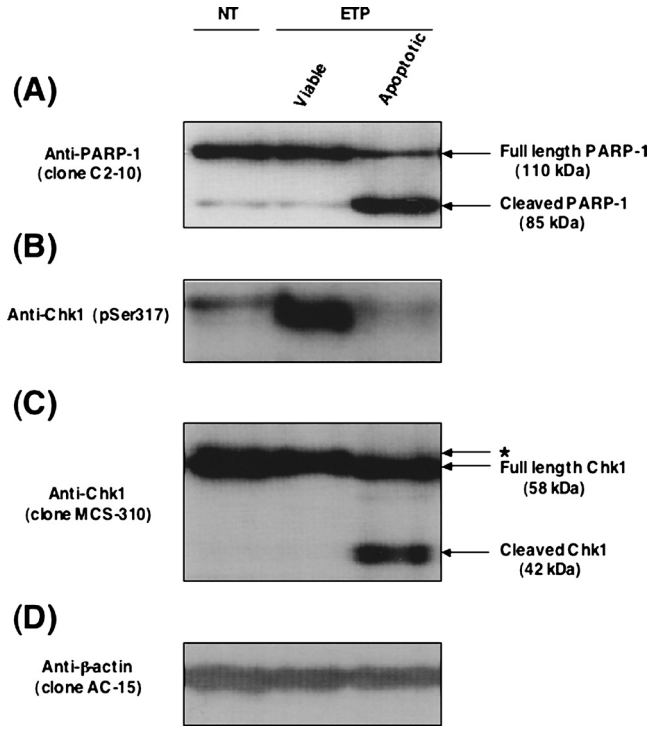

Fig. 1. Chk1 Is Cleaved during ETP-Induced Apoptosis

HeLa S3 cells were treated with vehicle or ETP of viable dose (5 μM) or apoptotic dose (100 μM) for 48 h. Proteins in the cell lysates were analyzed by Western blotting with indicated antibodies. Apoptosis and DNA damage under the viable and apoptotic conditions were monitored by PARP-1 cleavage (A) and phosphorylation of Chk1 at Ser317 (B), respectively. Western blotting with anti-Chk1 (clone MCS-310) is shown (C), and β-actin was used as loading control (D). Asterisk indicates an unidentified band, which may be from post-modified Chk1.

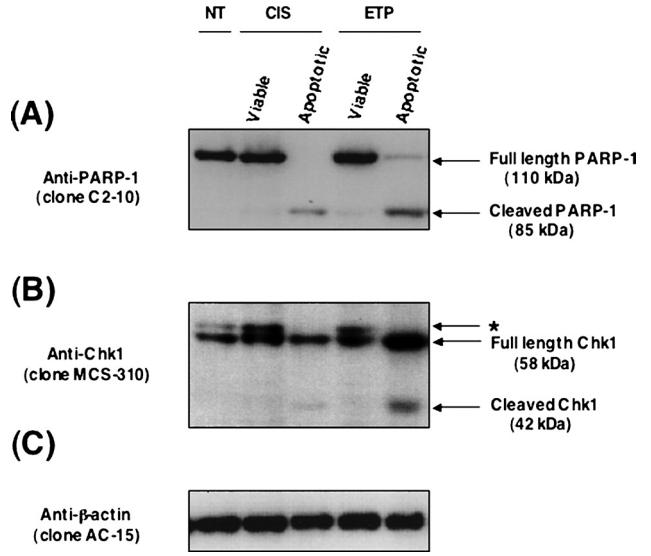

Fig. 2. Chk1 Is Also Cleaved during CIS-Induced Apoptosis

HeLa S3 cells were treated with vehicle or CIS of viable dose (10 μM) or apoptotic dose (60 μM) for 48 h. The cell lysates were subjected to Western blot analysis using antibodies as follow; (A) anti-PARP-1; (B) anti-Chk1 (clone MCS-310); (C) anti-β-actin. Asterisk indicates an unidentified band.

tem (ATTO) by using Supersignal West Femto Maximum Sensitivity Substrate (PIERCE).

RESULTS

It is known that genotoxic agents such as ETP and CIS induce growth arrest or apoptosis in a dose dependent manner. In this report, HeLa S3 cells were used to examine the mechanism involved in genotoxic stress-induced apoptosis. HeLa S3 cells were treated with 5 μM (viable dose) or 100 μM (apoptotic dose) ETP for 48 h, and total cell lysates were subjected to Western blotting with Chk1 antibodies (Fig. 1).

To confirm the viable and apoptotic conditions, PARP-1 cleavage was first examined, because cleaved PARP-1 is a reliable indicator of apoptosis, which is cleaved by caspase-3 or 7 from 110 kDa full length form into 85 and 25 kDa fragments during apoptosis.[19—21,25] Second, since it is known that phosphorylation of Chk1 at Ser317 is caused by various genotoxic stress,[1,2,26] DNA damage in the ETP-treatment was confirmed by the detection of Ser317-phosphorylation of Chk1. As shown in Fig. 1A, PARP-1 was clearly cleaved in the apoptotic dose of ETP-treated HeLa S3 cells. As expected, Chk1 phosphorylation at Ser317 was detected in the viable dose treatment of HeLa S3 cells (Fig. 1B). In contrast, in the ETP treatment of apoptotic dose, Ser317-phosphorylation of Chk1 could not be detected at 48 h, although it was observed after 6 h treatment (data not shown).

It is noteworthy that in Western blot analysis using mouse anti-Chk1 monoclonal antibody (clone MCS-310), another band below the full length Chk1 was detected under the apoptotic conditions, but not under the viable conditions (Fig. 1C). The fragment band appeared was approximately 42 kDa. In the treatment with viable (10 μM) or apoptotic dose (60 μM) of CIS, the same fragmentation of Chk1 was observed only in the apoptotic dose (Fig. 2). This fragmentation of Chk1 was also detected by Western blotting with goat anti-Chk1 polyclonal and rabbit anti-Chk1 polyclonal antibodies in ETP- or CIS-induced apoptotic HeLa S3 or HL-60 cells (data not shown). These results suggest that the unique 42 kDa fragment is generated by the cleavage of Chk1 during genotoxic stress-induced apoptosis. Furthermore, the results indicate that Chk1 cleavage is regulated in apoptosis-dependent pathways.

To prove this idea, we examined about the effect of an irreversible and cell-permeable pan-caspases inhibitor, z-VAD-fmk, on the cleavage of Chk1 in ETP-induced apoptosis. The treatment of z-VAD-fmk efficiently inhibited the cleavage of PARP-1 (Fig. 3A). This shows that the intracellular caspase activities are completely inhibited by z-VAD-fmk. Importantly, cleaved Chk1 was also completely diminished by z-VAD-fmk treatment (Fig. 3B). These results show that Chk1 is cleaved in a caspase-dependent pathway during ETP-induced apoptosis.

DISCUSSION

In this study, we demonstrated that genotoxic stress-induced apoptosis causes the cleavage of Chk1 in HeLa S3 cells. Induction of apoptosis by ETP or CIS as detected by PARP-1 cleavage is simultaneously accompanied by the cleavage of Chk1 into a fragment of 42 kDa. This is the first report to identify Chk1 as a substrate cleaved in genotoxic stress-induced apoptosis and may indicate the existence of a novel apoptotic pathway *via* Chk1 restricted cleavage.

Furthermore, we showed that the fragmentation of Chk1 is completely suppressed by the pan-caspase inhibitor, z-VAD-

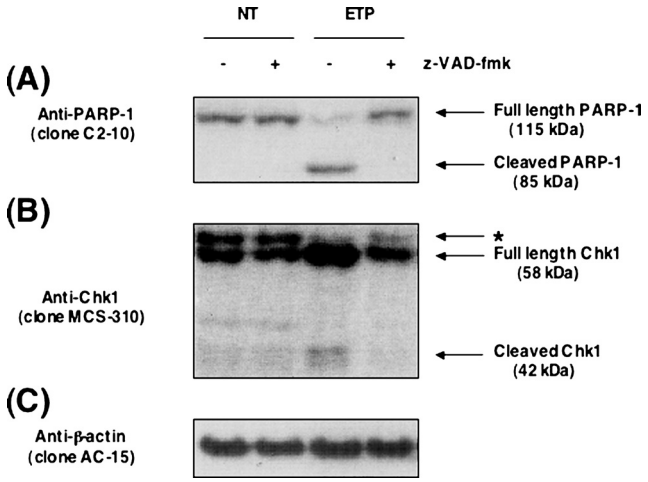

Fig. 3. z-VAD-fmk Inhibits Chk1 Cleavage in ETP-Induced Apoptosis

HeLa S3 cells were pretreated in the absence or presence of 100 μM z-VAD-fmk for 1 h and then etoposide was added into the medium at a final concentration of 100 μM. After another 48 h incubation, the cells were harvested and the cell lysates were analyzed by Western blotting with indicated antibodies (A, anti-PARP-1; B, anti-Chk1; C, anti-β-actin). Asterisk indicates an unidentified band.

fmk, treatment during genotoxic stress induced-apoptosis. Therefore, it is likely that a candidate catalyzing Chk1 cleavage is caspase family. It is known that Caspase-2 is more insensitive to z-VAD-fmk in identified caspase family.[24] Therefore, at least, Caspase-2 may not directly catalyze the cleavage of Chk1. We are getting some data to implicate about which caspases contribute to the Chk1 cleavage in the apoptotic conditions (unpublished data).

It has been shown that regulation of Chk1-protein level or kinase activity is important for cell cycle checkpoint.[10—12,16,17,26,27] In this paper, we show a possibility that there is a direct involvement of Chk1 in apoptosis regulation. Especially, Chk1 cleavage in a caspase-dependent pathway may be a crucial mechanism for apoptosis commitment. As illustrated in Fig. 4, genotoxic stress induces cell cycle arrest, and then cells try to repair. When the damage level is moderate, cells select to restart cell cycle after completing repair and Chk1-dephosphorylation by protein phosphatases[28—30] (right pathway). In fact, in cells treated with the viable dose of ETP for 48 h, Ser317-phosphorylation response of Chk1 continued until 48 h (Fig. 1B). On the other hand, when the damage level is severe, cells suspend repair, and then determine apoptosis initiation after Chk1-dephosphorylation (left pathway). As shown Fig. 1B, in the ETP-treatment of apoptotic conditions, Ser317-phosphorylation of Chk1 have already been diminished after 48 h treatment, although it was observed after 6 h treatment. These results may imply the existence of mechanisms that cells could suspend DNA repair machinery for apoptosis initiation or inactivate its for cell cycle restart.

There are two possibilities about when Chk1 is cleaved (Fig. 4). First is between repair abortion for apoptosis initiation and apoptosis execution. In this possibility, Chk1 may have a role as a determinant of cell survival or death. Second is during apoptosis execution. In this case, Chk1 cleavage may support efficient apoptosis execution. Now, we are investigating the period and the physiological roles of Chk1 cleavage during apoptosis.

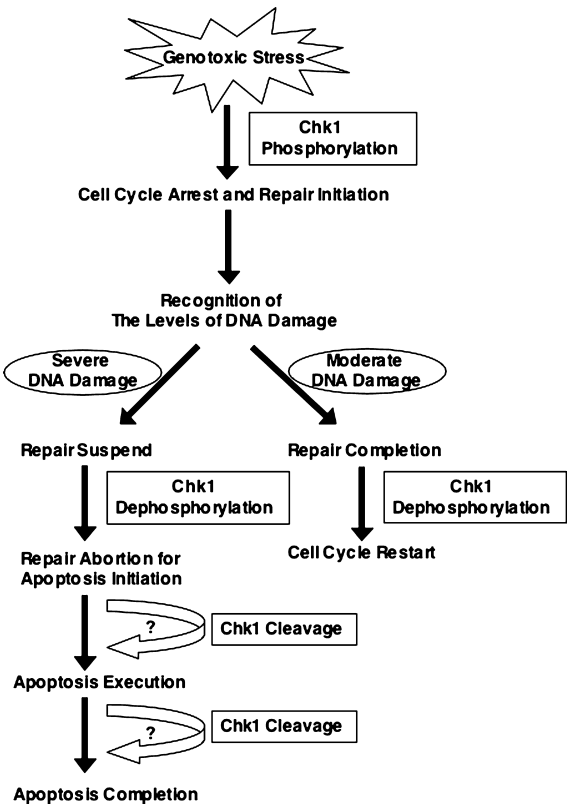

Fig. 4. A Hypothesis of Involvements of Chk1 Status in the Determination of Cell Fates, Repair or Apoptosis after DNA Damage

DNA damage induces checkpoint-mediated cell cycle arrest, and then cells try to repair. When the damage level is moderate, cells select to restart cell cycle after completing repair (right pathway). When the damage level is severe, cells abort repair and then undergo apoptosis (left pathway). The changes of Chk1 status are involved in the regulation of onset of these processes.

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