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-benzoylcarbonyl-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

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 at 37°C. Cells were seeded at a density of 1—2 × 10⁵ cells/cm² 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 WAKO and Peptide Institute, respectively. Rabbit anti-Chk1 polyclonal antibody and rabbit anti-Chk1 (pSer317) polyclonal antibody were obtained from Cell Signaling Technology. Mouse anti-α-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×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-
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. Second, since it is known that phosphorylation of Chk1 at Ser317 is caused by various genotoxic stress, 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 treatment of HeLa S3 cells (Fig. 1A). 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-
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-V AD-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 phosphatases28—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.

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