Cleavage-mediated Activation of Chk1 during Apoptosis*

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The Chk1 kinase is highly conserved from yeast to humans and is well known to function in the cell cycle checkpoint induced by genotoxic or replication stress. The activation of Chk1 is achieved by ATR-dependent phosphorylation with the aid of additional factors. Robust genotoxic insults induce apoptosis instead of the cell cycle checkpoint, and some of the components in the ATR-Chk1 pathway are cleaved by active caspases, although it has been unclear whether the attenuation of the ATR-Chk1 pathway has some role in apoptosis induction. Here we show that Chk1 is activated by caspase-dependent cleavage when the cells undergo apoptosis. Treatment of chicken DT40 cells with various genotoxic agents, UV light, etoposide, or camptothecin induced Chk1 cleavage, which was inhibited by a pan-caspase inhibitor, benzyloloxycarbonyl-VAD-fluoromethyl ketone. The cleavage of Chk1 was similarly observed in human Jurkat cells treated with a non-genotoxic apoptosis inducer, staurosporine. We have determined the cleavage site(s), Asp-299 in chicken and Asp-299 and Asp-351 in human cells. We further show that a truncated form of human Chk1 mimicking the N-terminal cleavage fragment (residues 1–299) possesses strikingly elevated kinase activity. Moreover, the ectopic expression of Chk1-(1–299) in human U2OS cells revealed an open kinase conformation, and the truncated Chk1 without the C-terminal half exhibited more than 20-fold higher activity than full-length Chk1 (16). Katsumura and Sagata (17) identified an autoinhibitory region (AIR) in the C-terminal 85 amino acids of *Xenopus* Chk1, which largely overlaps with a bipartite and unusually long Gln (SQ/TQ)-rich domain (14). The SQ/TQ domain is mapped between a highly conserved N-terminal kinase domain (residues 1–289) and a C-terminal domain with ill-defined function (15). The crystal structure of human Chk1 kinase domain (residues 1–289) revealed an open kinase conformation, and the truncated Chk1 without the C-terminal half exhibited more than 20-fold higher activity than full-length Chk1 (16). Katsumura and Sagata (17) identified an autoinhibitory region (AIR) in the C-terminal ~85 amino acids of *Xenopus* Chk1, which largely overlaps with a bipartite and unusually long nuclear localization signal (NLS). The authors further suggested that the phosphorylation of Ser-317 and Ser-345 induces a conformational change of the AIR and reverses the autoinhibition.

The Chk1 kinase is activated by phosphorylation of at least two serine residues, Ser-317 and Ser-345, located in a Ser/Thr-Gln (SQ/TQ)-rich domain (14). The SQ/TQ domain is mapped between a highly conserved N-terminal kinase domain (residues 1–265) and a C-terminal domain with ill-defined function (15). The crystal structure of human Chk1 kinase domain (residues 1–289) revealed an open kinase conformation, and the truncated Chk1 without the C-terminal half exhibited more than 20-fold higher activity than full-length Chk1 (16). Katsumura and Sagata (17) identified an autoinhibitory region (AIR) in the C-terminal ~85 amino acids of *Xenopus* Chk1, which largely overlaps with a bipartite and unusually long nuclear localization signal (NLS). The authors further suggested that the phosphorylation of Ser-317 and Ser-345 induces a conformational change of the AIR and reverses the autoinhibition.

The phosphorylation of Ser-345, but not Ser-317, is also required for proteolytic degradation of Chk1 following the treatment of the anti-cancer drug camptothecin (18). The Chk1 degradation is mediated by an ubiquitin/proteasome system containing Cul1 or Cul4A and was suggested to function in limiting the duration of Chk1 signaling induced by low-intensity replication stress. Furthermore, Claspin, a mediator protein required for Chk1 activation, is also degraded by an ubiquitin/proteasome system in an SCP3^P^T^C^P^-dependent manner (19, 20).

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2 The abbreviations used are: ATRIP, ATR-interacting protein; BRCT, BRCA1 C-terminal; AIR, autoinhibitory region; NLS, nuclear localization signal; FACS, fluorescence-activated cell sorter; h, human; ch, chicken; Chaps, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; Z, benzyloloxycarbonyl; fmk, fluoromethyl ketone.
The proteasome-dependent down-regulation of Claspin appears to be implicated in cell cycle-dependent fluctuation of its cellular level as well as recovery from genotoxic stress.

Robust genotoxic insults induce apoptosis rather than the cell cycle checkpoint. The implication of Chk1 in apoptosis remains to be elucidated. In the literature, some components in the ATR-Chk1 pathway are known to be cleaved by activated caspases during apoptosis. Human Rad9 is cleaved by caspase-3 after exposure to DNA-damaging agents or staurosporine (21). Cleavage-resistant Rad9 generated by site-directed mutagenesis appeared to protect the cells from death induced by DNA-damaging agents, suggesting a positive role of Rad9 cleavage in promoting apoptosis. Clarke et al. (22) have reported that Claspin is also cleaved by caspase-7 during the initiation of apoptosis, and a smaller C-terminal fragment has a dominant inhibitory effect on Chk1 phosphorylation. More recently, another group has shown that Claspin is cleaved into multiple fragments by caspase-3 and -7 and also degraded via the proteasome (23). The authors suggest the possibility that the down-regulation of Claspin by two different pathways promotes apoptosis. However, the biological roles of caspase-mediated cleavage of ATR-Chk1 components during apoptosis remain to be fully understood.

Here, we demonstrate that Chk1 is cleaved into two fragments by caspase during apoptosis in chicken DT40 and human Jurkat cells. The cleavage site(s) is Asp-299 in chicken and Asp-299 and Asp-351 in human cells, which are located between the N-terminal kinase domain and the C-terminal domain containing the AIR and NLS. We further show that a truncated form of human Chk1 mimicking the N-terminal cleavage fragment (residues 1–299) possesses ~8-fold higher kinase activity compared with full-length Chk1. Moreover, ectopic expression of Chk1-(1–299) in human U2OS cells induces locally condensed nuclear morphology as well as H2AX phosphorylation, suggesting a possible role of Chk1 cleavage in promoting apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Cycle Analysis**—Chicken B-lymphocyte line DT40 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma), 1% chicken serum, 50 μM β-mercaptoethanol, penicillin, and streptomycin (Invitrogen) in a 5% CO₂ incubator at 37 °C. Cells were treated with 50 μM etoposide (Sigma) or 500 nM camptothecin (Sigma) or irradiated with 30 J/m² of UV-C light from germicidal lamps (residues 1–299) possesses 8-fold higher kinase activity compared with full-length Chk1. Moreover, ectopic expression of Chk1-(1–299) in human U2OS cells induces locally condensed nuclear morphology as well as H2AX phosphorylation, suggesting a possible role of Chk1 cleavage in promoting apoptosis.

**Exponentially growing**

**DT40** or Jurkat cells (5 × 10⁵) were washed with phosphate-buffered saline and resuspended in 500 μl of growth medium. Twenty μg (DT40) or 50 μg (Jurkat) of expression plasmids were added to the cell suspension and electroporated using a Gene Pulser apparatus (Bio-Rad) at 250 V and 950 microfarads. After incubation for 18 h, cells were treated with or without apoptosis-inducing agents and lysed in lysis buffer (10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were clarified by centrifugation and used for immunoblotting analysis. The antibodies used in this study are as follows: monoclonal anti-Chk1 (G-4) and polyclonal anti-c-Myc (A-14) (Santa Cruz Biotechnology), polyclonal anti–Myc (Clontech), polyclonal anti-β-actin (Cell Signaling), and monoclonal anti-caspase-6 (Medical & Biological Laboratories). Monoclonal antibodies against DDB1 or glyceraldehyde-3-phosphate dehydrogenase were generated as described previously (24). The signals were visualized using SuperSignal West Femto maximum sensitivity substrate (Pierce) or Immobilon western chemiluminescent horseradish peroxidase substrate (Millipore) and a LAS1000 lumino-image analyzer (Fuji Film).
In Vivo Caspase Assay—DT40 cells were electroporated with pCMV-Myc/hChk1-Myc-His and lysed following an 18-h incubation. The lysates were incubated for 1 h at 37 °C with the active recombinant caspase-3, -6, -7, or -8 (Medical & Biological Laboratories) in caspase buffer (50 mM Hepes (pH 7.2), 50 mM NaCl, 10 mM EDTA, 0.1% Chaps, 5% glycerol, and 10 mM dithiothreitol). Proteins were separated in a SDS-polyacrylamide gel and immunoblotted with monoclonal anti-Myc antibody.

In Vivo Chk1 Kinase Assay—HEK293T cells were transfected with pCMV-Myc/hChk1 (wild-type, D130A, or residues 1–299) using TransFectin Lipid Reagent (Bio-Rad) and lysed after a 48-h incubation. The lysates were immunoprecipitated using polyclonal antiserum against c-Myc and protein A/G PLUS-agarose (Santa Cruz Biotechnology). After sequential washing with kinase buffer, phosphate-buffered saline, and kinase buffer (50 mM Hepes, 8 mM dithiothreitol, and 10 mM β-glycerophosphate), the immunoprecipitants were incubated in 20 μl of kinase buffer containing 100 μM biotinylated CHKtide (Upstate), 100 μM ATP, and 10 μCi of [γ-32P]ATP for 30 min at 30 °C. Kinase reaction was stopped by adding 5 μl of 500 mM EDTA, and biotinylated CHKtide was recovered by streptavidin-coated Dynabeads M-280 (Dynal). After extensive washing with kinase buffer, radioactivity incorporated into the CHKtide was measured using a liquid scintillation counter (Aloka).

Indirect Immunofluorescence Microscopy—U2OS cells were transfected with pCMV-Myc/hChk1 (wild-type, residues 1–299, or 1–299/D130A) by Effectene Transfection reagent (Qiagen) and incubated in a 5% CO2 incubator at 37 °C for 24 h. After fixation with methanol/acetone at −20 °C, the cells were immunostained with polyclonal anti-Myc and monoclonal anti-γ-H2AX (Upstate) antibodies, subsequently with Alexa Fluor 594 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugates (Invitrogen), and counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen). To obtain fluorescence images, a Leica DMIRBE microscope equipped with a cooled CCD camera (CoolSNAP HQ, Photometrics) was used.

RESULTS

Apoptosis Induces Chk1 Truncation in Chicken DT40 Cells—We have previously established a conditional DDB1 knock-out DT40 clone (DDB1−/−/hDDB1), which lacks all DDB1 alleles and contains human DDB1 transgene under control of the tetracycline-responsive element. The addition of doxycycline leads to complete loss of DDB1 and thereby induces a severe growth defect and subsequently cell death by apoptosis (24). During the analyses of various cellular factors under DDB1-depleted conditions, anti-Chk1 (G-4) monoclonal antibody revealed a time-dependent decrease of full-length Chk1 and concomitant appearance of a faster migrating band around 35 kDa following 96- and 120-h doxycycline treatment (Fig. 1A), when a significant population of DDB1−/−/hDDB1 cells undergoes apoptosis (24). Another anti-Chk1 (FL-476) polyclonal antibody also detected this band (data not shown), indicating an intrinsic signal of Chk1. The ~35-kDa band was similarly observed in DDB1−/−/hDDB1 cells treated with UV light (Fig. 1B), topoisomerase I poison camptothecin, and topoisomerase II poison etoposide (Fig. 1C) under doxycycline-free conditions. These results suggest that the truncated form of Chk1 is generated during apoptosis caused by DDB1 depletion as well as genotoxic treatment.

To ascertain the relationship between Chk1 truncation and apoptosis, we treated wild-type DT40 cells with various concentrations of etoposide (EPT) for 3 h and analyzed by immunoblotting with anti-Chk1 (G-4) or anti-β-actin antibody (A) or flow cytometry after fixation and propidium iodide staining (B). C and D, time-dependent induction of Chk1 truncation and apoptosis. Wild-type DT40 cells were treated with 50 μM etoposide for 1–3 h in the presence or absence of 50 μM Z-VAD-fmk (Z-VAD) and analyzed as described above.
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FIGURE 3. Chk1 is cleaved into two fragments by active caspase during apoptosis. A, ectopically expressed chicken Chk1 is cleaved into two major fragments following etoposide treatment. Wild-type DT40 cells were electroporated with either plasmid expressing chChk1-Myc-His (chChk1-MH) or Myc-chChk1-Myc-His-His (M-chChk1-MH) and incubated with or without 50 μM etoposide (ETP) for 3 h. Cell lysates were prepared and used for immunoblotting ([8] with anti-Myc (top), anti-Chk1 (G-4) (middle), or anti-β-actin (bottom) antibody. B, chicken Chk1 is cleaved by caspase-7. Wild-type DT40 cells were electroporated with a plasmid expressing Myc-chChk1-Myc-His and lysed after 18 h. The cell lysates were incubated with the active recombinant caspase-3, -6, -7, or -8 for 1 h at 37 °C and analyzed by immunoblotting with anti-Myc or anti-Chk1 (G-4) antibody (lanes 1–5). The lysates from DT40 cells that had been electroporated with a plasmid expressing Myc-chChk1-Myc-His and treated with 50 μM etoposide for 3 h was also loaded as a positive control (lane 6).

time course between the appearance of truncated Chk1 and sub-G₁ cells (Fig. 2, C and D). Moreover, pan-caspase inhibitor Z-VAD-fmk completely abolished Chk1 truncation as well as the increase of sub-G₁ population. Taken together, these results strongly indicate that genotoxic stress-induced Chk1 truncation is associated with apoptosis induction.

Chicken Chk1 Is Cleaved at Asp-299 by Active Caspase during Apoptosis—We next tried to understand the molecular basis of Chk1 truncation during apoptosis. Wild-type DT40 cells were electroporated with a plasmid expressing chicken Chk1 tagged with Myc and His tags at the C terminus (chChk1-Myc-His) and treated with 50 μM etoposide. The ectopic expression of tagged Chk1 was verified by immunoblotting with anti-Chk1 (G-4) and anti-Myc antibodies (Fig. 3A, lane 3). Upon etoposide treatment, anti-Chk1 (G-4) antibody showed a more intense signal of truncated Chk1, although this band is invisible with anti-Myc antibody (Fig. 3A, lane 2 versus lane 4). On the other hand, anti-Myc antibody, but not anti-Chk1 (G-4) antibody, detected new doublet bands around 20 kDa. The upper band seems to be a phosphorylated form of the lower band because it is still detected with phosphatase treatment led to a single band (data not shown). These results strongly suggest that chicken Chk1 is cleaved at a single site in the process of apoptosis, generating ~35-kDa N-terminal and ~20-kDa C-terminal fragments. Consistent with this notion, double-tagged Chk1 containing an additional Myc tag at the N terminus (Myc-chChk1-Myc-His) exhibited a slightly larger product (~38 kDa) following etoposide treatment, which is also detectable with anti-Myc antibody (Fig. 3A, lane 4 versus lane 6).

To test the possibility that Chk1 is cleaved by caspase(s), we performed an in vitro caspase assay using the lysate prepared from DT40 cells expressing Myc-chChk1-Myc-His. Following incubation with active recombinant caspase-3, -6, -7, or -8, only caspase-7 exhibited truncated Chk1 with the same mobility as the etoposide-induced Chk1 fragment in DT40 cells expressing Myc-chChk1-Myc-His (Fig. 3B, lane 6). Furthermore, the caspase-7-induced Chk1 cleavage was totally abolished by the addition of Z-VAD-fmk to the reaction (Fig. 3C, lane 2 versus lane 4). We conclude that Chk1 is cleaved by active caspase, most likely caspase-7, during apoptosis.

We further tried to determine the cleavage site of Chk1 using site-directed mutagenesis (Fig. 4A). Based on the size of the cleavage product, we mutated three Asp sites (Asp-299, Asp-329, or Asp-336) to Ala individually in Myc-chChk1-Myc-His and tested for the etoposide-induced cleavage. As shown in Fig. 4B, the substitution of Asp-299 to Ala (D299A) resulted in no Chk1 cleavage, whereas other two mutants (D329A and D336A) showed a comparable cleavage with wild-type Chk1. Consistently, the lysate from DT40 expressing Myc-chChk1(D299A)-Myc-His showed no Chk1 cleavage after incubation with active recombinant caspase-7 (Fig. 4C, lane 2 versus lane 3). Furthermore, the ectopic expression of truncated Chk1, Myc-chChk1(1–299), exhibited identical mobility with the etoposide-induced cleavage fragment of Myc-chChk1-Myc-His (data not shown). These results demonstrate that apoptosis-dependent Chk1 cleavage takes place at Asp-299 in chicken DT40 cells.

Human Chk1 Is Cleaved at Asp-299 and Asp-351 upon Apoptotic Stress—We asked whether Chk1 cleavage also occurs in human cells undergoing apoptosis. Human B-lymphocyte line Jurkat cells were treated with 1 μM staurosporine, a nonselective protein kinase inhibitor, and apoptosis induction was verified by the cleavage of caspase-6 (Fig. 5A) as well as the increase of the sub-G₁ population (Fig. 5B). Under these conditions, anti-Chk1 (G-4) antibody detected two truncated products (Fig. 5A, lanes 2 and 3), which were not observed in the presence of Z-VAD-fmk (lanes 4 and 5), indicating that Chk1 is also cleaved in apoptotic human cells. We similarly constructed plasmids expressing double-tagged human Chk1 (Myc-hChk1-Myc-His), wild-type, or D299A mutant and electroporated them into Jurkat cells. Following staurosporine treatment, wild-type Chk1 exhibited two cleavage fragments (Fig. 5C, lane 2 versus lane 4).
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human cells, we also constructed a plasmid expressing D351A mutant Chk1, based on the estimated size as well as the fact that Asp-351 is not conserved in chicken. As expected, Jurkat cells transiently expressing D351A mutant Chk1 showed only one product with higher mobility following staurosporine treatment (Fig. 5C, lane 6). Taken together, these results clearly indicate that Chk1 is cleaved at Asp-299 and/or Asp-351 in human cells undergoing apoptosis.

The N-terminal Cleavage Fragment of Chk1 Exhibits Elevated Kinase Activity and Induces Abnormal Nuclear Morphology as Well as H2AX Phosphorylation—It has been reported previously that Xenopus Chk1 contains the AIR at the C terminus, which suppresses its N-terminal kinase activity (17). Interestingly, the common cleavage site Asp-299 is mapped between the N-terminal kinase domain and the C-terminal AIR (Fig. 4A), prompting us to compare the kinase activities between full-length Chk1 and truncated Chk1 mimicking the N-terminal cleavage fragment (residues 1–299). HEK293T cells were transfected with a plasmid expressing N-terminally Myc-tagged human Chk1: wild-type Myc-hChk1, kinase-dead mutant Myc-hChk1 (D130A), or truncated mutant Myc-hChk1 (1–299). The equal amounts of Chk1 were immunoprecipitated with anti-Myc antibody, and their kinase activities were measured in vitro using a Chk1 substrate (CHKtide) and 

FIGURE 4. Caspase-mediated cleavage of chicken Chk1 takes place at Asp-299. A, a schematic representation of Chk1 domains and potential cleavage sites (Asp 299, Asp 329, or Asp 336) by caspase is shown. B, Chk1 is cleaved at Asp-299 in chicken DT40 cells treated with etoposide. Wild-type DT40 cells were electroporated with a plasmid expressing Myc-chChk1-Myc-His (M-chChk1-MH), wild-type (wt), or D299A, D329A, or D336A mutant. After 18 h, the cells were treated with 50 μM etoposide for 3 h and analyzed by immunoblotting (IB) with anti-Myc or anti-Chk1 (G-4) antibody. C, Chk1(D299A) mutant is not cleaved by active recombinant caspase-7. The lysate prepared from DT40 cells transiently expressing My-c-hChk1-Myc-His, wild-type, or D299A mutant was incubated with active recombinant caspase-7 in the presence or absence of 50 μM Z-VAD-fmk for 1 h at 37°C and analyzed by immunoblotting with anti-Myc or anti-β-actin antibody.

FIGURE 5. Human Chk1 is cleaved at Asp-299 and/or Asp-351 in Jurkat cells treated with staurosporine. A and B, Chk1 cleavage is observed in human cells treated with staurosporine (STS). Jurkat cells were treated with 1 μM staurosporine in the presence or absence of 50 μM Z-VAD-fmk for 6 or 12 h and analyzed by immunoblotting with anti-Chk1 (G-4) antibody (top), anti-caspase-6 (middle), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; bottom) antibody. C, Chk1 cleavage by staurosporine was analyzed by flow cytometry after fixation and propidium iodide staining (IB). C, Chk1 is cleaved at Asp-299 and/or Asp-351 in human Jurkat cells treated with staurosporine. Jurkat cells were electroporated with a plasmid expressing Myc-hChk1-Myc-His (M-hChk1-MH), wild-type (wt), or D299A or D351A mutant. After 18 h, the cells were treated with 1 μM staurosporine for 6 h and analyzed by immunoblotting (IB) with anti-Myc or anti-glyceraldehyde-3-phosphate dehydrogenase antibody.
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Phosphorylation with localized chromatin condensation. Furthermore, those cells were merged with strong signals of phosphorylated H2AX (γ-H2AX), whereas most of the cells transfected with full-length Chk1 or kinase-dead truncated Chk1-(1–299) displayed no γ-H2AX signals. These results raise a possibility that the N-terminal cleavage fragment of Chk1 might be implicated in enhancing apoptotic signals.

DISCUSSION

Chk1 has critical roles in G2/M- and S-phase checkpoints induced by genotoxic or replication stress and activated via the phosphorylation at Ser-317 and Ser-345 by ATR kinase in concert with several mediator proteins. On the recovery from cell cycle checkpoints after removal of the stress, the activated ATR-Chk1 pathway is down-regulated by proteasomal degradation of its components such as Chk1 and Claspin (18–20). The ATR-Chk1 signaling pathway is down-regulated when the caspase-dependent Chk1 cleavage. Consistently, caffeine, a phosphoinositide 3-kinase-related protein kinase inhibitor, showed no significant effects on Chk1 cleavage in DT40 cells treated with etoposide (data not shown).

Another important aspect would be subcellular localization of the N-terminal Chk1 fragment, which loses not only the C-terminal AIR but also the NLS. The immunostaining study using truncated Chk1-(1–299) mimicking the N-terminal fragment revealed a unique subcellular localization, in which Chk1-(1–299) is hardly merged with 4,6-diamidino-2-phenylinidole staining, whereas full-length Chk1 and kinase-dead truncated Chk1-(1–299/D130A) were detected mainly in the nucleus (Fig. 6B). More intriguingly, U2OS cells expressing truncated Chk1-(1–299) exhibited locally condensed nuclear morphology and phosphorylation of histone H2AX on Ser-139 (Fig. 6B). The H2AX phosphorylation is known to be triggered by DNA double-strand breaks, stalled replication forks, or single-stranded DNA gaps during nucleotide excision repair in quiescent cells (28–30).
Apoptotic DNA fragmentation also induces H2AX phosphorylation (31). Our data suggest that the N-terminal Chk1 fragment may induce DNA damage, probably double-strand breaks, enhancing apoptotic signals, or promote some apoptotic reactions thereby causing DNA fragmentation. Further study is required to clarify the mechanism of H2AX phosphorylation caused by the N-terminal Chk1 fragment and uncover a possible function of Chk1 cleavage in apoptosis induction. In summary, this study provides a new finding that Chk1 is activated by caspase-dependent cleavage during apoptosis and raises the possibility that the resultant N-terminal fragment may play a role in promoting apoptotic reactions through phosphorylating some substrates by its highly elevated kinase activity.

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