Depletion of Chk1 Leads to Premature Activation of Cdc2-cyclin B and Mitotic Catastrophe*

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Mitotic catastrophe occurs as a result of the uncoupling of the onset of mitosis from the completion of DNA replication, but precisely how the ensuing lethality is regulated or what signals are involved is largely unknown. We demonstrate here the essential role of the ATM/ATR-p53 pathway in mitotic catastrophe from premature mitosis. Chk1 deficiency resulted in a premature onset of mitosis because of abnormal activation of cyclin B-Cdc2 and led to the activation of caspases 3 and 9 triggered by cytoplasmic release of cytochrome c. This deficiency was associated with foci formation by the phosphorylated histone, H2AX (γH2AX), specifically at S phase. Ectopic expression of Cdc2AF, a mutant that cannot be phosphorylated at inhibitory sites, also induced premature mitosis and foci formation by γH2AX at S phase in both embryonic stem cells and HCT116 cells. Depletion of ATM and ATR protected against cell death from premature mitosis. p53-deficient cells were highly resistant to lethality from premature mitosis as well. Our results therefore suggest that ATM/ATR-p53 is required for mitotic catastrophe that eliminates cells escaping Chk1-dependent mitotic regulation. Loss of this function might be important in mammalian tumorigenesis.

Initiation of mitosis in mammals is triggered by the abrupt activation of cyclin B-Cdc2. Activation of Cdc2 is regulated by a complex process that includes the binding to its regulatory subunit, cyclin B, whose level rises at late S phase, and peaks in mitosis (1–4). Just before mitosis, most cyclin B-Cdc2 complexes exist in an inactive state because of the inhibitory phosphorylation of Cdc2 on Thr-14 and Tyr-15, whose phosphorylation is catalyzed by Wee1 and Myt1, and whose dephosphorylation is catalyzed by Cdc25 phosphatases.

Mitotic catastrophe was first identified in certain mutant fission yeast strains as a lethal phenotype characterized by gross abnormalities in chromosome segregation during mitosis (5, 6). A similar lethal phenotype was also observed in mammalian cells and found to result from premature mitosis (7, 8) or a failure to undergo complete mitosis (9, 10). However, there is still no broadly accepted definition of the term “mitotic catastrophe,” presumably because the major processes involved have not been described in molecular and genetic terms. For example, some researchers have argued that mitotic catastrophe is fundamentally different from apoptosis (11) because overexpression of anti-apoptotic genes including Bcl-2 and MDR1 can actually enhance the frequency of catastrophic mitosis (12, 13). In contrast, a recent study demonstrated that mitotic catastrophe induced by DNA damage is dependent on caspase activation, suggesting that it constitutes a special case of apoptosis (14, 15).

In response to blocks to DNA replication, eukaryotes activate checkpoint pathways that prevent genomic instability. In yeast models, spontaneous chromosomal breaks and gross chromosomal rearrangements have been detected in mutant strains that cannot respond to stalled or incomplete DNA replication (16, 17). Thus, impaired DNA replication checkpoint function in mammals likely causes gross chromosomal rearrangements, leading to the transformation of normal cells into cancer cells unless the cells undergoing premature mitosis are eliminated by cell death.

In mammals, incomplete DNA replication is detected by sensors and the signal is then relayed to kinases such as ATR, which phosphorylate and activate yet other kinases, such as Chk1. ATR- and Chk1-deficient mice die at an early embryonic stage of development with gross morphological abnormalities of their cell nuclei, suggesting the occurrence of mitotic catastrophe (18–20). Very recently, a study using conditional Chk1 knock-out mice also revealed that Chk1 deficiency caused severe cell death in proliferating somatic cells (21). However, the mechanism(s) by which the cell death is triggered and regulated remains elusive.

We report here the role of ATM/ATR-p53 in mitotic catastrophe induced by premature mitosis. Either Chk1 depletion or ectopic expression of Cdc2AF effectively caused DNA damage, specifically at S phase. The resulting DNA damage activated the ATM/ATR-dependent DNA damage checkpoint and subsequently stabilized p53 protein. Reduction of ATM/ATR proteins by their siRNAs2 or depletion of p53 significantly reduced lethality from premature mitosis.

MATERIALS AND METHODS

Cell Lines and Antibodies—Cells of the mouse embryonic stem (ES) cell line, EX226, and HCT116 cells were maintained as described (19, 22). Antibodies used were as follows, α-Chk1 (sc-7898, Santa Cruz), α-Cdc2 (sc-747, Santa Cruz), α-phospho-Cdc2 Tyr-15 (number 9111, Cell Signaling), α-cyclin B1 (sc-245, Santa Cruz), α-Chk2 (sc-748, Santa Cruz), α-Chk1 (sc-751, Santa Cruz), α-phospho-Tyr (number 9711, Cell Signaling), α-phospho-Ser-10 histone H3 (06–570, Upstate), α-Chk2 (sc-9064, Santa Cruz), α-phospho-caspase-3 (number 9661, Cell Signaling), α-caspase-9 (number 9504, Cell Signaling), α-cytoplasmic phosphohistone H3; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; TUNEL, TdT-mediated dUTP-biotin nick end labeling; PurA, Purvalanol A; DAPI, 4’,6-diamidino-2-phenylindole.

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2 The abbreviations used are: siRNA, small interfering RNA; ES, embryonic stem; pHH3, phosphohistone H3; Z-VAD, benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone; TUNEL, TdT-mediated dUTP-biotin nick end labeling; PurA, Purvalanol A; DAPI, 4’,6-diamidino-2-phenylindole.
To elucidate the mechanism(s) by which Chk1 deficiency results in premature mitosis, we first generated a cell system in which mitotic catastrophe is regulated. For this purpose, we generated conditional Chk1-deficient ES cells. Genomic DNA from individual ES clones was digested with NdeI and subjected to Southern hybridization with the 5′ probe indicated by the small bar below the map (Δ). The leftmost lane contains molecular size makers. C, immunoblot analysis of Chk1 or actin (control). D, growth defect in Chk1+/− ES cells. Numbers of Chk1+/− and Chk1lox/lox ES cells were counted at the indicated times after infection with Ad-Cre. Data are means ± S.D. of three separate experiments. E, impaired checkpoint functions in Chk1+/− ES cells. Chk1+/− and Chk1lox/lox ES cells were treated with 2.5 mM hydroxyurea (HU) or 10 gray of x-ray. After treatment, cells were harvested at the indicated hours and immunostained with anti-pHH3 antibody. Data are means ± S.D. of three independent experiments.

RESULTS AND DISCUSSION

Chk1 Deficiency Results in Premature Mitosis through Premature Activation of Cyclin B-Cdc2—To elucidate the mechanism(s) by which mitotic catastrophe is regulated, we first generated a cell system in which...
which premature mitosis is synchronously inducible. We generated conditional Chk1-deficient mouse ES cells by flanking the second exon of one Chk1 allele with loxP sites and disrupting the other allele by gene targeting (Fig. 1A). The lox-flanked (“floxed”) Chk1 allele could thus be converted to a null allele by excision of exon 2, which contains the translational initiation sequence and encodes the ATP binding site of the kinase, through the action of Cre recombinase. The construction of the floxed allele was confirmed by the appearance of an 11-kb NdeI fragment on Southern blot hybridization with a probe generated from a sequence 5′ of the targeted region (Fig. 1B). Infection of the Chk1lox/− ES cells with an adenovirus encoding Cre recombinase (Ad-Cre) resulted in a marked decrease in the amount of Chk1 protein within 12 h after infection and its almost complete disappearance after 24 h (Fig. 1C), indicating that the floxed fragment of the Chk1 allele was removed (yielding the Δ allele) in most cells within 24 h after infection. A similar infection of Chk1+/− cells had no effect on the abundance of Chk1. Infection with an adenovirus encoding β-galactosidase verified that >95% of ES cells became infected under our experimental conditions (data not shown). Whereas Chk1+/− cells continued to increase in number during 96 h of culture, the Chk1Δ− cells exhibited a pronounced growth defect (Fig. 1D), indicating that Chk1 is essential for ES cell proliferation. We next examined the DNA damage and DNA replication checkpoint functions in Chk1Δ− cells. Chk1lox/− and Chk1Δ− cells were treated with hydroxyurea (2.5 mM) or X radiation (10 gray), and their mitotic indices were measured. As expected, cell cycle arrest before mitosis induced by hydroxyurea (stalled replication forks) or X-radiation (DNA damage) was abolished in Chk1Δ− ES cells. The cytosolic and mitochondrial fractions from Chk1+/− and Chk1Δ− ES cells were subjected to immunoblotting using the indicated antibodies. E, changes in the mitochondrial membrane potential in Chk1Δ− ES cells. Chk1+/− and Chk1Δ− ES cells were stained with JC-1 in the absence or presence of Z-VAD. Fluorescence intensity was analyzed by flow cytometry.

Fluorescence-activated cell sorter analysis revealed that, in the case of Chk1Δ− cells, >95% of those that were phosphohistone H3 on Ser-10 (pH3)-positive, an M-phase marker, had a 4N DNA content. With Chk1Δ− cells, however, 70% of the pH3-positives had a DNA content of less than 4N (Fig. 2A). Microscopic laser-scanning cytometry revealed that chromosomal condensation was also observed in pH3-positive Chk1Δ− cells with a DNA content of less than 4N (Fig. 2B). Quantitation of chromosome condensation in individual cells revealed that >90% of Chk1Δ− cells with condensed chromosomes had a DNA content of less than 4N (Fig. 2C). Taken together,
these results clearly indicated that Chk1 deficiency results in the onset of mitosis before completion of DNA replication.

We next examined the effect of Chk1 depletion on mitotic inducers. The abundance of cyclin B1 and Cdc2 in Chk1Δ−/− ES cells did not vary after Ad-Cre infection (Fig. 2D). Phosphorylation of Cdc2 on Tyr-15, however, was markedly reduced at 36 h and undetectable at 48 h after Ad-Cre injection of Chk1Δ−/− cells (Fig. 2D). Consistent with this observation, the activity of cyclin B1-Cdc2 was up-regulated. In contrast, cyclin A-dependent kinase activity was not affected by Chk1 depletion, although the extent of tyrosine phosphorylation was slightly decreased (Fig. 2D).

**Cell Death Induced by Premature Mitosis Is a Form of Caspase-dependent Apoptosis Triggered by Cytochrome c Release—**Premature mitosis induced by Chk1 depletion led to cell death as a result of mitotic catastrophe. We next examined whether this cell death was due to apoptosis. The number of TUNEL-positive cells was markedly higher among Chk1Δ−/− cells than among Chk1+/− cells (Fig. 3A). The cell death was almost completely blocked by the addition of the caspase inhibitor, Z-VAD (Fig. 3B). Z-VAD itself did not affect cell cycle progression (data not shown). These results suggested that cell death induced by Chk1 depletion is because of caspase-dependent apoptosis.

Activated forms of caspases 3 and 9 were readily detected in Chk1Δ−/− cells but not in Chk1+/− cells (Fig. 3C). We next asked whether Chk1 depletion caused cytoplasmic release of cytochrome c, which triggers Apaf-1/caspase 9 complex and initiates caspase 9 activation (25). Cytochrome c was readily detected in the cytoplasmic fraction from Chk1Δ−/− cells even in the presence of Z-VAD (Fig. 3D). Loss of mitochondrial membrane potential, indicated by a left shift in JC-1 fluorescence, was also observed in Chk1Δ−/− cells (Fig. 3E). Taken together, these results suggested that apoptosis induced by premature mitosis is dependent on mitochondrial events.

**Chk1 Deficiency Causes DNA Damage during S Phase in a Manner Dependent on Active Cyclin B-Cdc2—**To determine whether apoptosis induced by premature mitosis is because of DNA damage, we examined the foci formation of phospho-H2AX (γH2AX), a robust marker for cellular DSBs (26). An increase in the amount and foci formation of γH2AX was observed after Chk1 depletion (Fig. 4A) as well as after IR irradiation used as a control. In addition, FACS analysis showed the increased population of cells with DNA content of less than 2N (Fig. 4B). These results suggested that premature mitosis induced by Chk1 depletion causes DNA damage, possibly DSBs. In yeast, DNA replication checkpoint kinases stabilize DNA replication fork progression during S
Checkpoin-dependent Mitotic Catastrophe

FIGURE 5. Ectopic expression of Cdc2AF mimics Chk1-depletion. A, ectopic expression of Cdc2AF induced premature mitosis. Cell cycle profiles of ES cells expressing either LacZ or Cdc2AF were analyzed by FACScan. B, increased Cdc2 protein after infection of adenoviruses expressing Cdc2AF. Cells were infected with adenoviruses expressing Cdc2AF and then harvested at the indicated times. The cell lysates were subjected to immunoblotting using anti-Cdc2 antibodies. C, cell death induced by Cdc2AF was reduced by the treatment with Z-VAD. Cell cycle profiles of cells infected with adenoviruses expressing Cdc2AF in the absence or presence of Z-VAD. Populations of the sub-G1, fraction are shown. D, activation of the Chk2-dependent DNA damage checkpoint in cells expressing Cdc2AF. The cell lysates from Chk1−/− and Chk1+/- ES cells were subjected to immunoblotting using the indicated antibodies. E, Cdc2AF expression caused DNA damage. IR-treated Chk1−/− ES cells or cells expressing either Cdc2AF or LacZ were double-stained with DAPI and antibodies specific to γH2AX. Bar, 2 μm. F, ectopic expression of Cdc2AF caused apoptosis specifically at S phase. Cells were infected with adenoviruses expressing either LacZ or Cdc2AF, synchronized at M phase by nocodazole for 12 h, and then released into G1 phase. Cell cycle profiles of cells expressing either LacZ (LZ) or Cdc2AF (AF) were determined by FACScan analysis. The level of Cdc2 protein was determined by immunoblotting using anti-Cdc2 antibody G.

FIGURE 6. Role of ATM/ATR-p53 in mitotic catastrophe induced by premature mitosis. A, ectopic expression of Cdc2AF induced premature mitosis in HCT116 cells. Cell cycle profiles of HCT116 cells expressing either LacZ or Cdc2AF were analyzed by FACScan. B, foci formation of γH2AX in wild-type (WT), Chk2−/−, and p53−/− HCT116 cells expressing Cdc2AF but not LacZ. Cells infected with adenoviruses expressing either LacZ or Cdc2AF were double-stained with DAPI and antibodies specific to γH2AX. Bar, 2 μm. C, reduction of ATM and ATR protein by their specific siRNA. HCT116 cells were transfected with the indicated siRNAs and harvested at 48 h after transfection. The cell lysates were then subjected to immunoblotting using the indicated antibodies. D, reduction of ATM and ATR proteins prevents cell death induced by premature mitosis. HCT116 cells were transfected with siRNAs as in C and infected with adenoviruses expressing LacZ (black bar) or Cdc2AF (white bar). Cells were harvested at 48 h after infection. The sub-G1 populations were then determined by FACScan analysis. E, reduction of ATM and ATR proteins diminished p53 stabilization in cells expressing Cdc2AF. Wild-type HCT116 cells were transfected with the indicated siRNAs and infected with adenoviruses expressing Cdc2AF. The cell lysates were then subjected to immunoblotting using anti-p53 antibodies and β-actin antibodies as a control. F, p53-dependent mitotic catastrophe induced by premature mitosis. Wild-type and p53−/− HCT116 cells were infected with adenoviruses expressing either LacZ (black bars) or Cdc2AF (white bars). Cells were harvested 48 h after infection and sub-G1 populations were determined by FACScan.

phase (27, 28). Thus, it is possible that the DNA damage caused by Chk1 depletion was because of a destabilized DNA replication fork and not the premature activation of cyclin B-Cdc2. To clarify this point, we first asked whether cell death induced by Chk1 depletion could be prevented by the inhibition of active cyclin B-Cdc2. Chk1-deficient cells were synchronized at the G1/S boundary and released into S phase. The increase in the sub-G1 population during S phase was significantly reduced by treatment with Purvalanol A (29), an inhibitor of cyclin B-Cdc2, but was unaffected by nocodazole (Fig. 4C). Although Purvalanol A has also been reported to inhibit cyclin A-Cdk2 with lower efficiency when compared with cyclin B-Cdc2, 10 μM Purvalanol A in the culture medium inhibited the initiation of mitosis but did not affect the initiation and progression of DNA replication in wild-type ES cells, suggesting that this reduction of sub-G1 population is likely because of inhibition of cyclin B-Cdc2 activity. More importantly, reduction of cyclin B1 protein by a specific siRNA significantly suppressed cancer cell death induced by Chk1 reduction (Fig. 4D) or ectopic expression of Cdc2AF (see below) (Fig. 4E). Thus, the cell death appeared to require active cyclin B-Cdc2, suggesting that the DNA damage from premature mitosis was because of the premature activation of cyclin B-Cdc2 and not to the loss of Chk1 function per se.

We then determined the phases of the cell cycle at which DNA damage is induced by active cyclin B-Cdc2. Chk1-depleted cells were synchronized at M phase and then released into G1 phase. Cells were harvested at the indicated times, and the cell cycle and foci formation by ATM and ATR proteins were determined by FACScan analysis. E, reduction of ATM and ATR proteins prevented cell death induced by premature mitosis. HCT116 cells were transfected with siRNAs as in C and infected with adenoviruses expressing LacZ (black bar) or Cdc2AF (white bar). Cells were harvested at 48 h after infection. The sub-G1 populations were then determined by FACScan analysis. D, reduction of ATM and ATR proteins diminished p53 stabilization in cells expressing Cdc2AF. Wild-type HCT116 cells were transfected with siRNAs as in C and infected with adenoviruses expressing Cdc2AF. The cell lysates were then subjected to immunoblotting using anti-p53 antibodies and β-actin antibodies as a control. F, p53-dependent mitotic catastrophe induced by premature mitosis. Wild-type and p53−/− HCT116 cells were infected with adenoviruses expressing either LacZ (black bars) or Cdc2AF (white bars). Cells were harvested 48 h after infection and sub-G1 populations were determined by FACScan.

B-Cdc2, suggesting that the DNA damage from premature mitosis was because of the premature activation of cyclin B-Cdc2 and not to the loss of Chk1 function per se.
PurA treatment reversed this failure (Fig. 4G). Foci formation by γH2AX was significantly increased beginning 9 h after termination of nocodazole treatment at which point cells entered into S phase (Fig. 4H). It is noteworthy that PurA treatment reduced foci formation by γH2AX. There results suggested that premature activation of cyclin B-Cdc2 at S phase but not at M to G1 phases caused DNA damage.

Activation of the DNA damage checkpoint in response to DNA damage has been shown to be dependent on ATM/ATR and subsequent stabilization of p53 protein (30). We therefore asked whether Chk1 depletion would activate this response. The protein level of p53 was significantly increased in Chk1−/− cells (Fig. 4I), and modification of Chk2 was readily detected after Chk1 depletion (Fig. 4I). These results suggested that premature mitosis induced by Chk1 depletion activates the DNA damage checkpoint response.

Ectopic Expression of Cdc2AFAF Mimics Chk1 Deficiency in ES Cells—Because DNA damage induced by Chk1 deficiency was almost completely suppressed by inhibition of cyclin B-Cdc2, we determined whether ectopic expression of Cdc2AFAF caused DNA damage during S phase progression. As expected, ectopic expression of Cdc2AFAF induced premature mitosis in ES cells (Fig. 5A). The level of Cdc2 protein expressed was increased almost 2-fold starting from 12 h after infection of the adenovirus expressing Cdc2AFAF (Fig. 5B), indicating that the expression level of Cdc2AFAF protein was almost the same as that of endogenous Cdc2. The ectopic expression of Cdc2AFAF effectively induced apoptosis that was inhibited by treatment with Z-VAD (Fig. 5C). As with Chk1 deficiency, immuno blotting revealed that ectopic expression of Cdc2AFAF caused the Chk2 band to shift upward and increased the level of p53 protein (Fig. 5D). Foci formation by γH2AX was also observed in ES cells expressing Cdc2AFAF as well as in cells exposed to IR (Fig. 5E). It is noteworthy that Cdc2AFAF caused DNA damage specifically at S phase and not at other phases of the cell cycle (data not shown) and thereby induced apoptosis (Fig. 5F), although the level of Cdc2AFAF was relatively constant (Fig. 5G). These results suggested that ectopic expression of Cdc2AFAF mimics a Chk1-deficiency in ES cells.

Ectopic Expression of Cdc2AFAF Induces Premature Mitosis in HCT116 Cells—It has been previously reported that in HeLa cells nuclear localization of cyclin B as well as dephosphorylation of Cdc2 at inhibitory sites is also required for mitotic entry after DNA damage (31, 32). During S phase and G2, cyclin B1 accumulates in the cytoplasm, and, just before mitosis, the majority of the cyclin B-Cdc2 complexes suddenly appear in the nucleus. However, in embryonic mouse cells, nuclear accumulation of cyclin B is also regulated by the activation of Cdc2 (33). Therefore, it is possible that ectopic expression of Cdc2AFAF triggers nuclear accumulation of cyclin B followed by induction of premature mitosis in somatic cells. The ectopic expression of Cdc2AFAF in human colon cancer HCT116 cells effectively increased the number of pHH3-positive cells with a DNA content of less than 4N, suggesting the induction of premature mitosis (Fig. 6A). In addition, foci formation by γH2AX was readily detected in wild-type, Chk2−/−, and p53−/− HCT116 cells expressing Cdc2AFAF (Fig. 6B), suggesting that foci formation occurs in a manner independent of functional Chk2 and p53 in mitotic catastrophe. These results demonstrated that the premature mitosis induced by Cdc2AFAF in HCT116 cells did not require either Chk2 or p53.

Role of ATM/ATR-Chk2-p53 in Mitotic Catastrophe—Because focus formation by γH2AX after ionizing radiation is dependent on ATM and ATR (34), the above data suggested that these kinases are active in cells undergoing mitotic catastrophe. We therefore determined the role of these kinases in apoptosis. Wild-type HCT116 cells expressing Cdc2AFAF were transfected with specific siRNAs for these kinases, and the change in population of the sub-G1 fraction was determined. Significant reductions of ATM and ATR proteins were detected after transfection of their specific siRNAs (Fig. 6C). A significant increase in this population was observed in wild-type HCT116 cells after expression of Cdc2AFAF. It is noteworthy that reduction of ATM and ATR proteins diminished this effect (Fig. 6D). Stabilization of p53 protein in HCT116 cells expressing Cdc2AFAF was also diminished by the reduction of ATM and ATR proteins, suggesting that ATM/ATR function upstream of p53 (Fig. 6E). The increase in the sub-G1 population was significantly diminished in p53−/− HCT116 cells, indicating that p53 plays an important role in apoptosis from premature mitosis (Fig. 6F). Although Chk2 appeared to be induced in the regulation of p53-dependent apoptosis because of DSBs in mouse (35, 36), apoptosis induced by premature mitosis in Chk2−/− HCT116 was almost the same as that in wild-type cells (data not shown). In this regard, it has also been reported that Chk2 is not involved in p53 regulation in human cells (37). Thus, the requirement for Chk2 in p53 regulation might be species-dependent. These results clearly indicate the critical role of the ATM/ATR-p53 pathway in cell death induced by mitotic catastrophe even though developmental defects in Chk1-null mice could not be prevented by p53 deletion, presumably because of a loss of an essential function of Chk1 in DNA replication independent of p53 (Fig. 3B).

In conclusion, Chk1 deficiency resulted in a premature onset of mitosis because of abnormal activation of cyclin B-Cdc2 and led to the caspase-dependent apoptosis. This abnormal activation of cyclin B-Cdc2 was associated with DNA damage, showing foci formation of γH2AX, specifically at S phase. Although the mechanism by which DNA damage is induced by active cyclin B-Cdc2 remains elusive, our present results suggest that mitotic catastrophe induced by premature mitosis is, at least in part, dependent on the ATM/ATR-p53 pathway.

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