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Depletion of the Pre-RC Proteins Induces Chk1/Chk2 Independent Checkpoint Responses and Apoptotic Cell Death in HeLa Cells

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Abstract: The initiation of eukaryotic DNA replication requires assembly of the pre-replicative complex (Pre-RC) through the concerted action of Orc, Cdc6, Cdt1 and Mcm2-7 complex during G1 phase. The pre-RC assembly licenses individual replication origins for the initiation of DNA replication and sufficient number of the pre-RC is essential for proper progression of S phase. However, it is not well known how cells recognize the completion of the pre-RC assembly before G1-S transition. In order to understand the cellular responses to the defects in pre-RC assembly, we depleted the known components of pre-RC proteins using the small interference RNAs in HeLa cells. Although the defects of pre-RC assembly by the depletion of the pre-RC proteins such as Orc2, Cdt1, Mcm2 & Mcm10 did not elicit the activation of Chk1- or Chk2-dependent checkpoint pathways, these cells still showed significant decrease in the cellular level of Cdc25A proteins. These results suggests that a novel checkpoint pathway exist in HeLa cells, which is not dependent upon Chk1 or Chk2 proteins and play essential roles in the cellular responses to the defects in the pre-RC assembly. Also, among those four proteins tested in this study, the depletion of Mcm10 and Cdt1 proteins significantly increased the apoptotic cell death in HeLa cells, suggesting that these proteins not only play roles in the pre-RC assembly, but also are involved in the checkpoint responses to the defects in the pre-RC assembly.

Key words: pre-replicative complex, checkpoint, Chk1, Chk2, apoptosis

For initiation of eukaryotic DNA replication, the pre-replicative complex (pre-RC) must be assembled on individual replication origins during G1 phase. The proteins involved in the initiation of DNA replication such as Cdc6, Cdt1 and minichromosome maintenance (Mcm) complex are sequentially recruited onto origin DNAs in an origin recognition complex (ORC)-dependent manner after mitosis (Bell and Dutta, 2002; Stillman et al., 1992). The binding of Cdc6 proteins on ORC is regulated by ATP hydrolysis (Randell et al., 2006) and Cdt1 binding is inhibited by Geminin (Li and Blow, 2005; Wohlschlegel et al., 2000). The binding of heterohexameric Mcm 2-7 complex onto replication origins finally licenses the pre-RC for initiation of DNA replication (Gillespie et al., 2001). The pre-RC complexes are activated during S phase by the action of two kinases, Cdc7 and Cdk2, and other initiation factors such as Mcm10, Cdc45 and GINS are recruited to origin DNAs and lead to the formation of replication fork machinery (Bell and Dutta, 2002; Gambus et al., 2006; Sawyer et al., 2004).

The Orc is a six subunit complex (Orc1 to Orc6) that recognizes the replication origin DNAs and acts as the platform of the pre-RCs (Bell and Dutta, 2002). Cdt1 protein is essential for the loading of Mcm complex onto replication origin, and the downregulation of Cdt1 after S phase by geminin is essential for prevention of re-replication (Li and Blow, 2005; Maiorano et al., 2005; Melixetian et al., 2004). The MCM 2-7 complex is a major candidate for the replicative DNA helicase that works, at the replication fork (Bailis and Forsburg, 2004; Bell and Dutta, 2002; Cho et al., 2006). Although all these proteins play essential roles in the initiation of DNA replication, several recent studies have suggested that they are also involved in other cellular responses such as mitosis, chromosome stability, and checkpoint responses. In yeast, mutation of *orc2* induced cell cycle arrest in G1-S or M phases as well as catastrophe in DNA replication (Gibson et al., 2006; Teer et al., 2006). Similarly, in Drosophila, *orc2* and *orc5* mutations caused defects in chromosome condensation and mitotic arrest

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(Pflumm and Botchan, 2001). Depletion of Orc2 protein by siRNA in human cells also resulted in aberrant chromosome structures and defects in mitotic progression (Prasanth et al., 2004), suggesting that Orc proteins also play important roles in mitosis. Deregulation of Cdt1 is reported to induce chromosomal damages and checkpoint responses (Fujita, 2006; Tada, 2007). Mcm proteins are also required for the S-phase checkpoint response and the loss of Mcm function generates DNA damage checkpoint responses (Bailis and Forsburg, 2004; Cortez et al., 2004; Forsburg, 2004; Ricke and Bielinsky, 2004).

The checkpoint pathways activated by DNA disorders has traditionally been divided into two major pathways. One is regulated by Ataxia-Telanglectasia mutated (ATM)/Chk2 kinase and the other is regulated by Ataxia-Telanglectasia and Rad-3-related (ATR)/Chk1 kinase (Abraham, 2001). ATM is activated by DNA double-strand breaks (DSBs), and the increase of ATM kinase activity induces p53-dependent and p53-independent pathways that eventually inhibit the activity of Cdk2/cyclin E (Abraham, 2001; Kaufmann and Paules, 1996). ATR is a chromatin binding protein kinase that is activated by UV irradiation or various chemicals inhibiting DNA replication such as hydroxyurea. Activated ATR bound to ATRIP phosphorylates and activates the checkpoint kinase Chk1. ATR-ATRIP complex activation during S phase downregulates Cdk activities and inhibits mitosis entry (Abraham, 2001; Bartek et al., 2004; Heffernan et al., 2002). The ATR-dependent pathway is also essential for the regulation of DNA replication during S-phase (Tercero et al., 2003), and the phosphorylation of Chk1 or Chk2 by ATM or ATR leads to degradation of Cdc25A and cell cycle arrest (Falck et al., 2001; Xiao et al., 2003).

Although cellular responses to defects in the pre-RC assembly is required for proper progression of S phase, the cellular mechanisms recognizing completion or defects in pre-RC assembly are not understood well. In this study, we depleted pre-RC proteins such as Orc2, Cdt1, Mcm2 and Mcm10 by small interference RNAs (siRNAs) in HeLa cells. From the analysis of cellular responses to the defects, we found that Chk1/Chk2-independent checkpoint pathway responding to the defects in pre-RC existed in HeLa cells and some pre-RC proteins might also be involved in this checkpoint pathway.

MATERIALS AND METHODS

Cell culture and Reagents

The human cervical adenocarcinoma cells HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics, and maintained at 37°C in a humidified incubator containing 5% CO_2. Hydroxylurea and staurosporine were purchased from Sigma (USA).

Transfection of siRNAs

The sense sequences of siRNAs were as followings: GL2 5'-AACGUACGCGGAAUACUUCGA-3', Orc2 5'-AAGA AGGAGCGAGCGCAGCUU-3', Cdt1 5'-AAAUUACCA GCUCACCAUCGA-3', Mcm2 5'-UCAUCGGAAUCCU UCACCA-3', Mcm10 5'-AAAGAGUUGCAAGAGGAA UUA-3'. All siRNA were chemically synthesized from Samchully Pharm (Daejeon, Korea). Transfections of siRNAs were carried out by using oligofectamine (Invitrogen, USA) and the manufacturer's protocol. In most experiments, siRNA was used at the concentration of 120 nM for transfection.

Propidium Iodide (PI) staining and Microscopic Analysis

PI is a highly polar dye impermeable to cells with preserved membranes. For the staining of cells that have lost membrane integrity, cells were washed once with PBS and incubated with PBS containing 10 μg/ml of PI. After incubation for 5 min at room temperature, the cells were examined under an inverted fluorescence microscope.

Immunoblotting and Antibodies

For immunoblotting, HeLa cells were harvested and washed with PBS. To prepare total cell lysate, the cells were resuspended with cell lysis buffer containing 50mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM leupeptin, 1 mM pepstatin, 1 mM benzamidine and 1 mM PMSF. The cells were disrupted by sonication and the amounts of proteins were measured by Bradford assay. Total proteins (30 μg per lane) were analyzed by SDS-PAGE followed by Western blotting. Antibodies against phospho-Chk1 (ser 317), phospho-Chk2 (Thr 68), Cyclin A, cleaved caspase 3 and Poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Co (USA). Anti-Mcm10, anti-Cdt1, anti-Chk1 and anti-Chk2 antibodies were purchased from Abcam Co (UK). Anti-Mcm2, anti-cyclin E, anti-Cdc25A and anti-actin antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-Orc2 antobody was purchased from BD Biosciences (USA), and Anti-cyclin B1 was purchased from Calbiochem (USA).

RESULTS

Depletions of the pre-RC proteins did not elicit Chk1/Chk2-dependent checkpoint responses

Inhibition of DNA replication during S phase or DNA damage in mammalian cells generally activates the Chk1/Chk2-dependent checkpoint pathways, which eventually lead to prevention of cell cycle progression or to apoptotic cell death (Bartek et al., 2004; Montagnoli et al., 2004). In order to examine whether the cells recognize the defect of

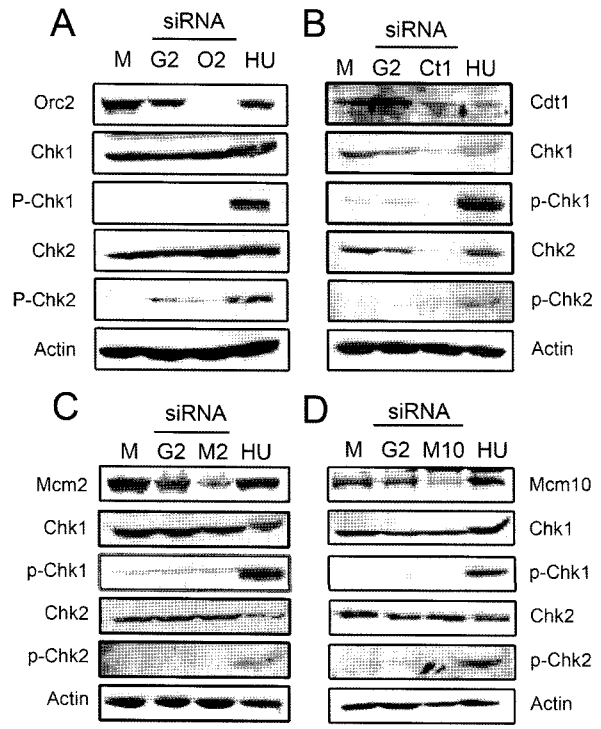

Fig. 1. Depletion of pre-RC proteins does not activate Chk1 or Chk2 kinasese. (A) HeLa cells were transfected with 120nM siRNA and incubated for 48h. Hydroxylurea was treated at the concentration of 5 mM for 20h (lane4). Cellular levels of phosphorylated Chk1 or Chk2 were determined by Western blotting using antibodies specific for phospho-Chk1 or Chk2. Lane M, Western blotting using mock treated cells; G2, GL2 siRNA transfected cells; O2, Orc2 siRNA transfected cells; HU, hydroxyurea-treated cells. Actin was used as a loading control. (B), (C) and (D) The same method as (A) was applied for Cdt1 (B), Mcm2 (C), and Mcm10 (D).

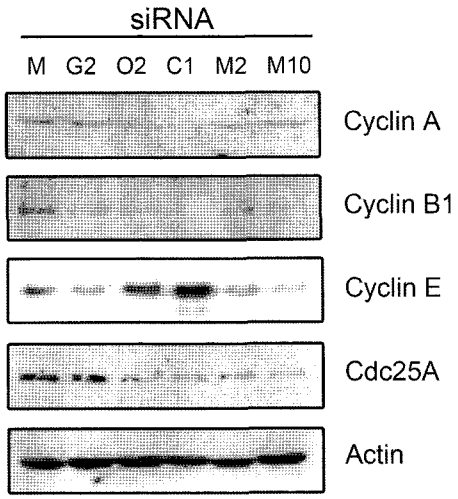

Fig. 2. Depletion of pre-RC proteins by siRNAs leads to down-regulation of Cdc25A. HeLa cells were transfected with 120nM siRNA and incubated for 48h. The cells were harvested and cellular levels of individual proteins were analyzed by Western blotting analysis. Lane M, mock transfected cells; G2, GL2 control siRNA transfected cells; O2, Orc2 siRNA; C1, Cdt1 siRNA; M2, Mcm2 siRNA; M10, Mcm10 siRNA. Actin was used as a loading control.

pre-RC assembly as DNA damage or replication block and activate the typical checkpoint responses, we depleted the known components of pre-RC proteins such as Orc2, Cdt1, Mcm2 and Mcm10 using siRNAs and examined the checkpoint responses. Although Mcm10 protein is not a core component of the pre-RC, the function of Mcm10 protein is required for the assembly of pre-RC. Therefore, depletion of Mcm10 protein would also result in defective pre-RC. HeLa cells transfected with siRNAs against these initiation factors significantly reduced the amount of respective proteins as shown in Fig. 1. Treatment with hydroxyurea stimulated phosphorylation of Chk1 and Chk2, which indicated activation of their kinase activities and Chk1/Chk2-dependent checkpoint pathways. On the other hand, depletion of the pre-RC proteins tested in this study did not cause the phosphorylation of Chk1 or Chk2 proteins, suggesting that the Chk1/Chk2 dependent checkpoint pathways are not activated by the defects in the pre-RC assembly.

Although the typical replication block and DNA damage checkpoint pathways are not activated by the depletion of pre-RC proteins, the cells still need to prevent cell cycle progression. Since the cells harboring defective pre-RC are not properly replicated during S phase, the progression of cell cycle in this condition would cause genomic instability. In order to examine the effects of defective pre-RC on cell cycle regulators, the cellular levels of cyclins and Cdc25A were determined by Western blotting after transfection of siRNAs against the pre-RC proteins (Fig. 2). The results showed no change in cyclin A and B, but that cycin E was significantly increased by the depletion of Orc2 or Cdt1. Cyclin E is a S-phase cyclin essential for the entry into S phase. The increase in cyclin E suggested that the cell cycle in Orc2 or Cdt1 depleted cells is arrested in S phase. On the other hand, the amount of cyclin E was not increased by the depletion of Mcm2 proteins, and depletion of Mcm10 resulted in a decrease in cyclin E. These differences in cyclin E levels against depletion of pre-RC proteins suggested that the effects to cyclin E are not a common response caused by defects in the pre-RC assembly. On the other hand, the cellular levels of Cdc25A decreased significantly by depletion of any of the pre-RC proteins tested in this study. Cdc25A is an essential cell cycle regulator, which is required for activation of S-cyclin Cdk and G1/S transition. Therefore, these results suggested that human cells might have a novel Chk1/Chk2-independent checkpoint pathway that is activated by defects in the pre-RC assembly.

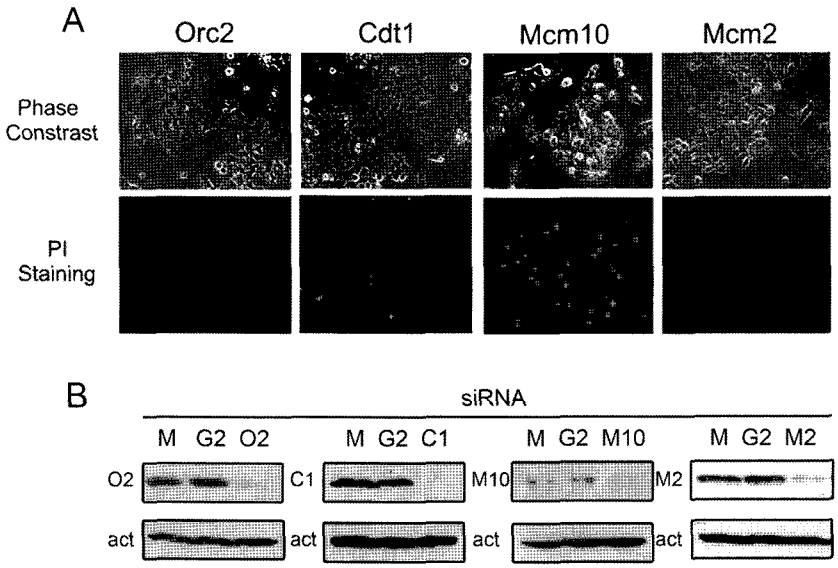

Fig. 3. Cell death induced by depletion of pre-RC proteins by siRNAs. (A) HeLa cells were transfected with 120nM siRNA. After 48h incubation, the cells were stained with propidium iodide and observed under a fluorescence microscope. Apoptotic cells displayed abnormal morphology and were stained by propidium iodide. (B) HeLa cells were transfected with 120nM siRNA and incubated for 48h. The cells were harvested and cellular levels of proteins were determined by Western blotting analysis. Lane M, mock transfected cells; G2, GL2 control siRNA transfected; O2, Orc2 siRNA; C1, Cdt1 siRNA; M10, Mcm10 siRNA; M2, Mcm2 siRNA. Actin was used as loading control.

Depletion of pre-RC proteins induced apoptotic cell death

Since the pre-RC assembly is essential for initiation of DNA replication, its defects may lead to aborted progression of S phase. Recently, depletion of known initiation factors such as Cdc6 or Cdc7 was shown to induce cell cycle arrest or cell death in some cancer cells (Lau et al., 2006; Montagnoli et al., 2004). In order to test whether depletion of pre-RC-related initiation factors induce cell death, we stained the siRNA-transfected cells with propidium iodide. As shown in Fig. 3, Mcm10 siRNA significantly increased cell death at 48 h after transfection, and Cdt1 siRNA also increased cell death, although the numbers of dead cells were relatively low. The cells incubated for 72 h after Mcm10 or Cdt1 siRNA transfction also showed significant levels of cell death (data not presented). We then tested whether this cell death occurred by apoptosis. For this, we examined the cleaved form of caspase-3 and poly (ADP-ribose) polymerase (PARP), which are the major indicators of apoptosis (Kumar, 2007; Okada and Mak, 2004). As shown in Fig. 4, cleaved caspase-3 (17 kDa and 19 kDa) and PARP were significantly increased by the depletion of Mcm10 and Cdt1. Although cleaved caspase-3 also appeared to increase by the depletion of Orc2 and Mcm2, an increase in PARP cleavage was observed only when Cdt1 or Mcm10 proteins were depleted. From these results, we concluded that the depletion of Cdt1 and Mcm10 proteins also induced apoptotic cell death in HeLa cells. These results suggested that these two proteins not only play roles in the initiation of DNA replication, but are also involved in the checkpoint

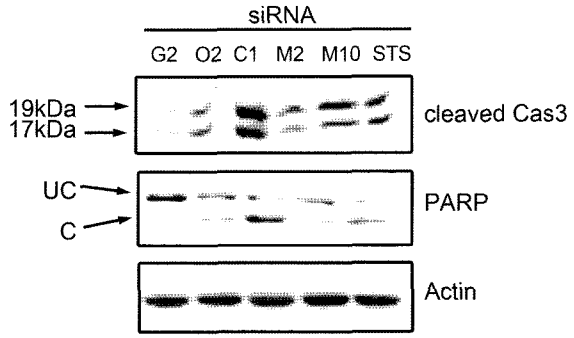

Fig. 4. Induction of apoptosis by depletion of pre-RC proteins. HeLa cells were transfected with 120 nM siRNA. After incubation for 48h, cleaved caspase-3 and PARP were examined by Western blotting analysis. Lane G2, GL2 siRNA transfected; O2, Orc2 siRNA; C1, Cdt1 siRNA; M2, Mcm2 siRNA; M10, Mcm10 siRNA. Actin was used as a loading control. UC, uncleaved form; C, cleaved form.

responses against defects in the pre-RC assembly.

DISCUSSION

The pre-RC must be established completely before G1/S transition in eukaryotic cells (Bell and Dutta, 2002; Stillman et al., 1992). Since the assemblies of pre-RCs on individual replication origins are essential for the initiation process, incomplete assembly or defects in pre-RC assembly may lead to abortion of S phase. Although many studies suggested that the DNA replication initiation factors are also involved in the checkpoint pathways (Lau et al., 2006; Montagnoli et al., 2004; Park et al., 2007; Prasanth et al.,

2004; Tada, 2007), the existence of checkpoint pathways recognizing the pre-RC formation has not been clear until now.

The checkpoint responses activated by DNA damages or replication blocks in S phase play essential roles for proper progression of cell cycle and maintenance of genome integrity, and ATR-dependent activation of Chk1 or ATM-dependent activation of Chk2 plays an essential role in these cellular responses. As shown in this study, depletion of the pre-RC proteins did not lead to activation of Chk1 or Chk2 kinases. Since single stranded DNA or double stranded DNA breaks appeared to be the main activator for ATR and ATM, these results may reflect that defects in pre-RC assembly caused by siRNAs do not lead to production of large single stranded regions or many strand breaks. However, even in the absence of Chk1 or Chk2 activation, all cells harboring the defects still showed a significant decrease in Cdc25A levels, implying activation of signaling pathway to arrest cell cycle. Although we still do not understand the mechanisms that recognize defects in the pre-RC assembly and that down-regulate major Cdk regulators such as Cdc25A, these results strongly suggested that Chk1/Chk2-independent checkpoint pathways responding to defects in the pre-RC exist in mammalian cells.

While depletions of any of the pre-RC related proteins tested in this study resulted in the reduction of Cdc25A level, a significant level of apoptotic cell death occurred by depletion of Mcm10. Also, induction of apoptosis, judged by caspase-3 cleavage and PARP proteins, was not caused by depletion of all pre-RC proteins. Therefore, induction of apoptotic cell death did not appear to be a common checkpoint response activated by defective pre-RC. Recently, many reports suggested that the proteins involved in the initiation of DNA replication also play additional roles during elongation, in checkpoint responses to DNA damages and in mitosis (Lau et al., 2006; Prasanth et al., 2004; Ricke and Bielinsky, 2004). Cdt1 has shown to be involved in the DNA damage checkpoint responses as a target of DNA damage-dependent and ubiquitin-dependent proteolysis (Higa et al., 2003; Hu et al., 2004). Mcm10 protein was also reported to play additional roles in the regulation of polymerase alpha and induction of mitotic checkpoint pathways (Ricke and Bielinsky, 2004). The apoptotic cell deaths caused by depletions of these two proteins might be caused by defects of their non initiation-related roles. However, we can not rule out the possibilities that defects of the pre-RC assembly caused by depletion of Mcm10 or Cdt1 proteins actually induced apoptotic cell death in HeLa cells. As shown in this study, our results clearly suggested that there is a novel Chk1 or Chk2-independent checkpoint pathway responding to defects in the pre-RC assembly. If depletion of these two initiation proteins also elicited apoptotic cell death, the proteins may also be involved in activation of the checkpoint responses. Although we described some evidence suggesting the existence of a novel checkpoint pathway recognizing defects in the pre-RC assembly, the exact roles and action mechanisms of this checkpoint pathway is not clear. Further studies should be carried out to clarify the nature of this novel checkpoint pathway sensing the assembly of pre-RC.

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