The Interaction between Checkpoint Kinase 1 (Chk1) and the Minichromosome Maintenance (MCM) Complex Is Required for DNA Damage-induced Chk1 Phosphorylation*

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Background: Chk1 is a key mediator protein that regulates replication checkpoint.

Results: Chk1 interacts with the MCM complex.

Conclusion: The MCM complex facilitates the recruitment of Chk1 onto chromatin.

Significance: This study provides mechanistic insights into DNA damage-induced phosphorylation of Chk1, the key step for checkpoint activation.

Chk1 is an essential mediator of the DNA damage response and cell cycle checkpoint. However, how exactly Chk1 transduces the checkpoint signaling is not fully understood. Here we report the identification of the heterohexamic minichromosome maintenance (MCM) complex that interacts with Chk1 by mass spectrometry. The interaction between Chk1 and the MCM complex was reduced by DNA damage treatment. We show that the MCM complex, at least partially, contributes to the chromatin association of Chk1, allowing for immediate phosphorylation of Chk1 by ataxia telangiectasia mutated and Rad3-related (ATR) in the presence of DNA damage. Further, phosphorylation of Chk1 at ATR sites reduces the interaction between Chk1 and the MCM complex, facilitating chromatin release of phosphorylated Chk1, a critical step in the initiation and amplification of cell cycle checkpoint. Together, these data provide novel insights into the activation of Chk1 in response to DNA damage.

Mechanisms that ensure the fidelity and timing of DNA replication in proliferating cells are critical in the maintenance of genome integrity and the prevention of tumorigenesis. Central to these genome surveillance pathways is a Ser/Thr protein kinase, Chk1. Chk1 regulates both DNA replication and replication checkpoint (1). Complete loss of CHK1 leads to embryonic lethality in mice (2, 3), whereas loss of one copy of this gene causes spontaneous cell death even in the absence of extrinsic stress (4), suggesting that Chk1 is essential for cell viability. The major function of Chk1 is to relay the DNA damage signal from the upstream kinase ATR to various downstream effectors through its phosphorylation at two Ser residues, Ser-317 and Ser-345, by ATR (5). As a result, activation of Chk1 will lead to cell cycle arrest or delay, gene transcription, damage repair, or cell death (1).

Recent studies suggest a spatiotemporal regulation model of Chk1, in which DNA damage induces ATR-dependent phosphorylation of Chk1 on chromatin followed by a rapid release of phospho-Chk1 from chromatin into soluble nucleus and the cytoplasm, where Chk1 activates the cell cycle checkpoints, as well as being degraded; the latter functions as an autoinhibitory mechanism to terminate the checkpoint signaling (6). In this regard, chromatin association of Chk1 is crucial for checkpoint initiation. However, a key question is how exactly does Chk1 associate with and dissociate from chromatin?

The MCM complex is the core component of eukaryotic DNA replication machinery and has recently been indicated as an important player in replication checkpoint (7–9). Here we report that human Chk1 associates with the MCM complex in unperturbed cells. DNA damage reduces the interaction between Chk1 and the MCM complex. The MCM complex partially contributes to chromatin association and phosphorylation of Chk1. Further, Chk1 phosphorylation at ATR sites reduces the interaction between Chk1 and the MCM complex, facilitating chromatin release of phospho-Chk1, which likely will contribute to subsequent checkpoint activation.

EXPERIMENTAL PROCEDURES

Cell Cultures, Transfection, Cell Proliferation, and Cell Death—HEK293 T, HeLa, U2-OS, and A549 cells were cultured in DMEM with 10% FBS. HEK293 T cells were transfected with calcium phosphate, while other cell lines were transfected with Lipofectamine 2000 (Invitrogen) or X-tremeGENE (Roche Applied Science) according to the manufacturer’s protocols. Immunoblotting, Immunoprecipitation, and Antibodies—Immunoblotting was carried out as described previously (10, 11). Anti-Chk1 (DCS-1310 and G4) and anti-MCM7 (141.2)
antibodies were from Santa Cruz. Anti-phospho-Chk1 (133.D3) antibodies were from Cell Signaling. Antibodies against human MCM2 were described previously (12). For immunoprecipitation, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml peptatin A, and 0.2% NP-40) for 30 min on ice. The lysates were sonicated (HEAT Systems, output 3) for 15 s on ice, and supernatants were collected by centrifugation. Under certain circumstances, the supernatants were treated with 2 units of micrococcal nuclease (New England Biolabs) for 15 min at 37 °C or 50 μg/ml ethidium bromide (EtBr), and antibodies were added (1 μg/1 mg lysates) and incubated at 4 °C overnight. Then 40 μl of protein A/G beads were added and incubated for an additional 2 h. The beads were washed and washed five times with lysis buffer, resuspended in 1× sample buffer, run on SDS-PAGE, and immunoblotted as mentioned in the figure legends.

**Plasmid Construction**—Myc- or GFP-tagged vectors expressing Chk1 WT or mutants were described previously (13). MCMs were generated using standard PCR with lentiviral vectors for each MCM (12) used as the template.

**Chromatin Fraction**—Cell fractionation was carried out as described previously (14). Cells were lysed in 100 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 mM sucrose, and 10% glycerol) containing 0.2% Triton X-100, 1 mM DTT, and protease inhibitors on ice for 8 min. The cell suspension was centrifuged at 1,000 rpm for 5 min at 4 °C. The supernatant contains the cytoplasmic fraction. The pellet was resuspended in 100 μl of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors), placed on ice for 30 min, and centrifuged (1,500 rpm for 5 min in a microcentrifuge at 4 °C), and the supernatant was combined with cytoplasmic fraction as the non-chromatin fraction. The pellet was resuspended in 150 μl of buffer C (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% NP-40, 1 mM DTT, and protease inhibitors) on ice for 10 min, briefly sonicated, and centrifuged (13,000 rpm for 10 min in a microcentrifuge at 4 °C). The supernatant was a chromatin-enriched fraction. Proteins in these fractions were quantitated and analyzed using SDS-PAGE.

**Identification of Chk1-interacting Protein by Mass Spectrometry**—U2-OS cells stably expressing FLAG-Chk1 were fractionated into cytoplasmic and nuclear compartments. Chk1-interacting proteins were isolated by FLAG M2 (Sigma) beads and eluted with 5 mg/ml 3×FLAG peptides in PBS. The eluate was first denatured in 8 M urea and then reduced and alkylated with 10 mM tris-(2-carboxyethyl)-phosphine hydrochloride (Roche Applied Science) and 55 mM iodoacetamide (Sigma-Aldrich), respectively. The samples were digested overnight with trypsin (Promega) and pressure-loaded onto a 250-μm silica capillary columns (Polymeric Technologies). After desalting, each biphasic column was connected to a 100-μm silica capillary analytical C18 column. Each MudPIT column was placed in line with an 1100 quaternary HPLC pump (Agilent Technologies), and the eluted peptides were electrosprayed directly into an LTQ mass spectrometer (Thermo Scientific). MS/MS spectra were extracted using RawXtract (version 1.9.9) (15) and searched with the SEQUEST algorithm (16) against a human International Protein Index (IPI) database (17). SEQUEST search results were assembled and filtered using the DTSelect (version 2.0) algorithm (18), requiring peptides to be at least half-tryptic and a minimum of two peptides per protein identification. The protein identification false positive rate was kept below 5%.

**RESULTS**

**Chk1 Interacts with the MCM Complex**—Recent studies demonstrated that Chk1 resides both in the nucleus and in the cytoplasm (6, 19). Our latest research showed that it is the nuclear, but not the cytoplasmic, pool of Chk1 that supports cell viability (13). To further understand the roles of Chk1 in cells, we sought to identify specific Chk1-interacting proteins in the nucleus. To this end, we generated a stable U2-OS cell line expressing FLAG-tagged human Chk1. Cytoplasmic and nuclear extracts from the stable clone or the vector control were immunoprecipitated with anti-FLAG M2 beads, eluted with 3×FLAG peptide, and subjected to mass spectrometric analysis (Fig. 1A).

The results showed that peptides corresponding to Chk1 were only identified in the FLAG-Chk1-expressing cells, but not from control cells. Peptide recovery of Chk1 from the nuclear and the cytoplasmic IPs was 32 and 30%, respectively, indicating a successful isolation of Chk1 and likely its associating proteins. We were able to identify some known Chk1-interacting proteins (Cul4A, DDB1, and Skp1 (6, 20)), but not others (Claspin and proliferating cell nuclear antigen (PCNA) (21, 22)), probably because different cell lines and experimental conditions were used. Among those proteins that were specifically identified from the nuclear Chk1 IP, the MCM complex appears to be most interesting to us due to its known roles in DNA replication and replication checkpoint. Further, all six components of the MCM complex (MCM2–7) were present exclusively in the nuclear, but not in the cytoplasmic, Chk1 IP with relatively high peptide sequence coverage (Fig. 1B), suggesting that this is not a nonspecific interaction with Chk1.

To confirm the mass spectrometry results, we performed Co-IP to determine the interaction between Chk1 and MCM proteins in cells. To do so, we overexpressed tagged Chk1 and each MCM protein in HEK293T cells. The results showed that overexpressed Chk1 interacted with all MCM proteins (Fig. 1, C–H). During IP, we sonicated the cell lysates and treated samples with micrococcal nuclease. This indicates that the interaction between Chk1 and the MCM complex is DNA-independent. To further confirm this idea, we performed Co-IP between Myc-Chk1 and HA-MCM7 (as an example of the MCM complex) by adding EtBr in the cell lysate to eliminate the impact of DNA on the Chk1/MCM interaction. The results showed that the presence of EtBr during Co-IP did not affect the interaction between Chk1 and MCM7 (Fig. 2A), supporting the notion that Chk1 interacts with MCM proteins independently of DNA. In our hands, the levels of overexpressed MCM proteins were 2–3-fold of that of endogenous proteins (data not shown). Further, we also detected interaction between endogenous Chk1 and endogenous MCM proteins (Fig. 2D). These results suggest that Chk1 associates with the MCM complex in unperturbed cells.
To understand whether Chk1 can directly interact with the MCM complex, we used GST-Chk1 bound on glutathione-agarose beads to pull down MCM proteins individually produced through in vitro transcription and translation. We detected an interaction between Chk1 and MCM2, MCM6, and MCM7, but not MCM3, MCM4, or MCM5 in vitro (Fig. 2B), indicating a multi-interface association between Chk1 and the MCM complex.

**FIGURE 1. Chk1 interacts with the MCM complex.** A, schematic diagram for purifying Chk1-interacting proteins. B, summary of mass spectrometry data for the MCM complex from the nuclear pool of Chk1-IP. Shown are unique peptide number and sequence coverage. C–H, differently tagged Chk1 and one of the six components of the MCM complex were transfected into HEK293T cells for 48 h; the cell lysates were sonicated and treated with 2 units of micrococcal nuclease (New England Biolabs) for 15 min at 37 °C, immunoprecipitated with the antibody against the tag of Chk1, and immunoblotted with antibodies against the tag for each individual MCM. The same membrane then was stripped and rebotted with antibodies against the tag for Chk1. WCE were also probed with the indicated antibodies for protein expression in cells.

**FIGURE 2. DNA damage reduces the interaction between Chk1 and the MCM complex.** A, HEK293T cells were transfected with Myc-Chk1 and HA-MCM7 for 48 h and lysed, and 50 μg/ml EtBr was added into the Co-IP reaction, followed by immunoblotting with the indicated antibodies. B, GST or GST-Chk1 proteins bound on glutathione-agarose beads were purified from bacteria and used to pull down MCM proteins produced by in vitro transcription and translation assays. MCM2, MCM6, and MCM7, but not MCM3, MCM4, and MCM5, were detected by GST-Chk1 beads. C, HEK293T cells were transfected with Myc-Chk1 for 48 h and treated or not with 2 mM HU for 4 h, and cell lysates were subjected to IP with anti-Myc antibodies followed by immunoblotting with anti-MCM2 antibodies. The same membrane was then stripped and rebotted with anti-MCM7 and anti-Myc antibodies. The relative intensity of co-immunoprecipitated endogenous MCM7 was analyzed by the National Institutes of Health ImageJ software. Protein expression in WCE was examined. D, HEK293T cells were treated with 500 nM camptothecin (CPT) for 4 h, immunoprecipitated with control IgG or anti-Chk1 antibodies, and immunoblotted with anti-MCM2 antibodies. The same membrane was then stripped and rebotted with anti-phospho-Chk1 and anti-Chk1 antibodies. The relative intensity of co-immunoprecipitated endogenous MCM2 was analyzed by the National Institutes of Health ImageJ software. E and F, HEK293T cells were transfected with tagged Chk1 and Cdc45 or GINS1, treated with 500 nM camptothecin (CPT) for 4 h, and immunoprecipitated as in C. The relative intensity of co-immunoprecipitated GFP-Cdc45 or GFP-GINS1 was analyzed by the National Institutes of Health ImageJ software. Protein expression in WCE was shown.
The Interaction between Chk1 and the MCM Complex Is Reduced by Replicative Stress—Subsequently, we asked whether DNA damage would affect the interaction between Chk1 and the MCM complex. To do so, we transfected HEK293T cells with Myc-Chk1, treated or not with a replicative stress agent, hydroxyurea (HU), and examined the interaction between Myc-Chk1 and endogenous MCM proteins. Because the six MCM proteins form a stable complex in cells, we reasoned that detection of two components (e.g. MCM2 and MCM7) suffices to indicate the presence of the entire MCM complex. Our results showed that the interaction between overexpressed Chk1 and endogenous MCM2 or MCM7 is reduced by HU treatment (Fig. 2C, lanes 2 and 3). We also show that the interaction between endogenous Chk1 and MCM proteins was reduced by another replicative stress, camptothecin, a topoisomerase 1 inhibitor (Fig. 2D, lanes 2 and 4).

The MCM complex, together with Cdc45 and the GINS (5-1-2-3) complex, form the CMG DNA helicase for DNA replication (23, 24). If Chk1 forms a protein complex with MCMs, it should also associate with Cdc45 and the GINS complex in cells. Our Co-IP data show that indeed Chk1 and endogenous MCM2 or MCM7 suffices to indicate the presence of the entire MCM complex. Our results showed that the interaction between overexpressed Chk1 and endogenous MCM2 or MCM7 is reduced by HU treatment (Fig. 2C, lanes 2 and 3). We also show that the interaction between endogenous Chk1 and MCM proteins was reduced by another replicative stress, camptothecin, a topoisomerase 1 inhibitor (Fig. 2D, lanes 2 and 4).

The C Terminus of Chk1 Is Mainly Responsible for Its Interaction with the MCM Complex—To determine which region of Chk1 interacts with the MCM complex, we transfected HEK293T cells with FLAG-MCM2 and Myc-tagged full-length Chk1 (FL), the N-terminal catalytic domain (N), or the C-terminal regulatory domain (C) for 48 h, lysed, and immunoprecipitated with anti-Myc antibodies, and then immunoblotted with anti-FLAG antibodies. The same membrane was stripped and rebotted with anti-MCM7 and anti-Myc antibodies. Protein expression in WCE is also shown. C, HEK293T cells were transfected with FLAG-MCM2, Myc-Chk1 WT, S317A, S345A, F380D, or G446N mutants for 48 h. The FLAG-MCM2 proteins were collected by the FLAG M2-agarose beads and used to pull down equal amounts of Myc-Chk1 WT or mutants from cell lysates (Input), and then immunoblotted as indicated. The relative intensity of the anti-Myc (for Chk1 WT or mutant proteins) blot was normalized to that of the anti-FLAG blot using the ImageJ software.

The C terminus of Chk1 contains two highly conserved motifs (CM1 and CM2) (Fig. 3A), which regulate the cellular localization of Chk1 (13). Mutating two residues (G446N and...
F380D) in these motifs not only led to cytoplasmic localization but also increased protein instability of Chk1 (13). To understand whether these mutants affect the interaction between Chk1 and the MCM complex, we transfected FLAG-MCM2, Myc-Chk1 WT, S317A, S345A, G446N, or F380D mutant in HEK293T cells separately. We collected FLAG-MCM2 on the FLAG M2-agarose beads and used the beads to pull down equal amounts of Myc-Chk1 WT or mutant proteins from the lysate. By doing so, we can avoid the cellular localization and protein instability issues of these two mutants. The results showed that indeed the G446N and F380D mutants displayed reduced interaction with MCM2 as compared with the Chk1 WT (Fig. 3C, lanes 2, 5, and 6), suggesting that these two residues might be important for the Chk1/MCM interaction. Interestingly, mutating Ser-345, the key ATR phosphorylation site for full activation of Chk1 (25), to Ala did not affect the interaction of Chk1 with MCMs (Fig. 3C, lanes 2 and 4), indicating that phosphorylation at ATR site is not required for the interaction of Chk1 with the MCM complex.

The MCM Complex Partially Contributes to Chromatin Association of Chk1—Research results from this group and others suggest that a substantial portion of Chk1 (21, 30–50% depending on the cell type) is located on chromatin in the absence of DNA damage (6, 19). Chromatin association of Chk1 is critical for checkpoint activation because it allows the rapid phosphorylation of Chk1 by ATR in response to DNA damage or replicative stress. However, how exactly Chk1 is recruited to chromatin remains unclear as Chk1 itself does not directly bind to chromatin (1). Known Chk1-interacting proteins, including Claspin and proliferating cell nuclear antigen, do not seem to recruit Chk1 onto chromatin because depletion of these two genes did not reduce the level of Chk1 associated on chromatin (21, 22). Our data showed that Chk1 interacts with the MCM complex and that this interaction is reduced by DNA damage. This led us to ask whether the MCM complex plays a role in the recruitment of Chk1 to chromatin.

To address this question, we used lentivirus-based shRNA targeting human MCM2 or MCM7 to inhibit expression of the MCM complex and determined expression of Chk1 in chromatin versus non-chromatin compartments. Despite a partial knockdown of MCMs (ranging from ~40 to 60% likely due to technical limitations including the efficacy of the shRNA and the essentiality of MCMs in cell viability), we repeatedly observed a clear reduction in the level of Chk1 on chromatin with a concomitant increase of Chk1 in the non-chromatin fraction (Fig. 4, A and B). This is unlikely due to cell cycle arrest or changes in total Chk1 levels by MCM depletion. First, depletion of MCM2 or MCM7 only had minimal effect on cell cycle profile (Fig. 4C), similar to results reported in HeLa and Drosophila cells (7). Second, inhibition of the MCM complex did not change the total cellular level of Chk1 (Figs. 4A and 5A). Third, when we used synchronized cell populations (e.g. cells synchronized at the G1/S boundary or in S phase), the level of Chk1 on chromatin fraction was still reduced in MCM-depleted cells as compared with control cells (similar data as in Fig. 4B, not shown). Together, these data suggest that the MCM complex did not change the total cellular level of Chk1 (Figs. 4A and 5A). Because chromatin association is important for Chk1 phosphorylation by ATR in response to DNA damage, we asked whether depletion of MCMs could reduce Chk1 phosphorylation by DNA damage because fewer Chk1 proteins are recruited to chromatin. We showed that this is indeed the case as depletion of MCM2 dose-dependently reduced Chk1 phosphorylation.
Chk1 Interacts with MCMs

FIGURE 5. Phosphorylation of Chk1 at ATR sites reduces its interaction with the MCM complex. A, A549 cells were infected with control or increasing doses of shMCM2 lentiviral particles for 3 days, treated with 2 mM HU for 4 h, and immunoblotted with the indicated antibodies. shMCM2 alone does not induce Chk1 phosphorylation. B, HEK293T cells were transfected with FLAG-MCM2 and GFP-Chk1 WT, G448D, L449R, or L449R/S345A mutant for 48 h, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-p-Ser-345 antibody of Chk1. The same membrane was stripped and rebotted with anti-FLAG and anti-GFP antibodies. Protein expression in WCE is shown. pChk1 phospho-Chk1. C, HEK293T cells were transfected with GFP-Chk1 WT or L449R for 48 h. Around 15% of cells were lysed as WCE, while the remaining cells were fractionated into chromatin versus non-chromatin fractions, and expression of phosphoryso-Chk1 (pChk1) was examined. The same membrane was stripped and reprobed with the anti-Chk1 antibodies. Note that endogenous Chk1 proteins behaved nearly equally in GFP-Chk1 WT or L449R mutant cells. D, proposed model. The question mark represents other putative factors that might also facilitate Chk1 chromatin association. The dashed arrow indicates checkpoint activation after Chk1 phosphorylation. See “Results” for details.

Phosphorylation of Chk1 at ATR Sites Reduces Its Interaction with the MCM Complex—Following its phosphorylation, Chk1 will be rapidly released from chromatin into soluble nucleus (1). Based on the findings presented above, we speculate that phosphorylation of Chk1 by ATR reduces its association with the MCM complex, leading to the release of Chk1 from chromatin. The observations that DNA damage reduced the interaction between Chk1 and the MCM complex (Fig. 2) and that the Chk1 S345A mutant showed a stronger interaction with MCMs than the Chk1 WT (Fig. 3C) support this hypothesis.

To address this issue, we took advantage of two constitutively active Chk1 mutants (G448D and L449R) that we previously identified, which undergo constitutive phosphorylation at ATR sites even in the absence of DNA damage (25). We predict that these two mutants would barely interact with the MCM complex because the majority of them are in the phosphorylated form (25). We transfected FLAG-MCM2 and GFP-Chk1 WT, G448D, or L449R mutant into HEK293T cells, immunoprecipitated with anti-FLAG M2 beads, and immunoblotted with anti-phospho-Ser-345-Chk1 antibodies. As reported previously (25), the G448D and L449R mutants, but not Chk1 WT, were phosphorylated at ATR sites under normal growth condition (Fig. 5B, the anti-phospho-Chk1 blot from whole cell extracts (WCE), lanes 3 and 5). Mutating the Ser-345 to Ala completely abolished the phospho-signal in the L449R mutant (Fig. 5B, the anti-phospho-Chk1 blot from WCE, lane 4). However, we did not detect any phospho-Chk1 from FLAG-MCM2 IP (Fig. 5B, the anti-phospho-Chk1 blot from IP). The same membrane was stripped and rebotted with anti-GFP antibodies. The results show that although the two Chk1 mutants were expressed at comparable levels with the WT (Fig. 5B, the anti-GFP blot from WCE), only the Chk1 WT, but not the two mutants, was detected from the FLAG-MCM2 IP (Fig. 5B, the anti-GFP blot from IP, lanes 2, 3, and 5).

Because G446N impaired the interaction between Chk1 and the MCM complex (Fig. 3C), the reduced interaction between L449R or G448D and MCMs might be due to changes of the same CM2 domain caused by the L449R or G448D mutation, but not due to Ser-345 phosphorylation of these two mutants. To address this issue, we examined the interaction of the Chk1 L449R/S345A double mutant with MCMs. The results showed that S345A mutation restored the interaction between Chk1 L449R and the MCM complex (Fig. 5B, the anti-GFP blot from IP, lanes 3 and 4). These results, together with our previous data that L449R did not induce cytoplasmic shuttling of Chk1 (13), suggest that the L449R mutation per se does not affect its interaction with MCMs, supporting the idea that phosphorylation at ATR sites of Chk1 reduces the interaction between Chk1 and MCMs.

If our hypothesis were true, we would predict that chromatin association of the Chk1 L449R mutant would be significantly reduced as compared with the Chk1 WT. Our data show that indeed the majority of the L449R mutant of Chk1 is in the non-chromatin cellular compartment (Fig. 5C). Together, these data suggest that phosphorylation of Chk1 at ATR sites inhibits its
interaction with the MCM complex, contributing to chromatin release of phospho-Chk1 after DNA damage treatment.

**DISCUSSION**

In this study we provide evidence for a novel interaction between Chk1 and the core component of DNA helicase, the MCM complex. We show that this interaction is important for chromatin association of Chk1, a critical step for the initiation of cell cycle checkpoint. Further, we propose that ATR-dependent phosphorylation of Chk1 reduces its interaction with the MCM complex, facilitating the rapid release of phospho-Chk1 from chromatin into soluble nucleus. These results illustrate a model that sheds significant light on DNA damage-induced Chk1 phosphorylation, and likely subsequent checkpoint activation (Fig. 5D).

Chk1 is a key checkpoint mediator whose phosphorylation is critical for cellular response to DNA damage or replicative stress, and ultimately, cell survival. ATR, the predominant kinase that phosphorylates Chk1 in cells (27), mainly localizes on chromatin (6). Thus, chromatin association is essential for Chk1 phosphorylation and the initiation of cell cycle checkpoints. Here we show that the MCM complex facilitated chromatin association of Chk1 and contributed to Chk1 phosphorylation. We only observed a partial chromatin release of Chk1 when the MCM expression was inhibited by shRNA. This could be due to the incomplete depletion of the MCM complex. However, we cannot preclude the possibility that other proteins also participate in the association of Chk1 with chromosomal DNA. Nevertheless, our data clearly illustrated an important role of the MCM complex in chromatin association and DNA damage-induced phosphorylation of Chk1.

Previously, it was reported that knockdown of MCM7 inhibited replication stress-induced Chk1 phosphorylation (7), which is consistent with our observation. However, depletion of MCM7 did not affect ionizing radiation-induced Chk1 phosphorylation (7). Several possibilities could lead to this discrepancy. (a) The first one may be related to different knockdown effects of MCMs between published literature (7) and our study. In our hands, the lentiviral shMCM2 significantly reduced the levels of both MCM2 and MCM7 proteins, suggesting the inhibition of the entire MCM complex. On the other hand, in the previous study, the specific siRNA against MCM7 barely reduced the level of MCM2 although MCM7 was significantly reduced (7). Therefore, it is likely that the remaining MCM7 was sufficient to form functional MCM complex that can still facilitate chromatin association of Chk1, which resulted in the lack of inhibition of Chk1 phosphorylation by ionizing radiation in that particular experiment. (b) As mentioned above, other factors might also contribute to the chromatin association and retention of Chk1 (Fig. 5D, question mark). This may become an issue especially when different types of DNA damage agents are used as they activate checkpoint and Chk1 phosphorylation (initiation and maintenance) through slightly different mechanisms. This might be exacerbated when different cell lines are used (the epithelial A549 line in our hands versus the osteoblast U2-OS in the previous study (7)). (c) As stated in the previous study (7), each subunit of the MCM complex may have unique functions that are not fully understood yet. Therefore, knockdown of different MCM proteins might display different phenotype.

Interestingly, the interaction between Chk1 and the MCM complex is reduced by DNA damage. This is in line with the notion that Chk1 undergoes chromatin release after DNA damage (1). Our data suggest that phosphorylation of Chk1 at ATR sites likely reduces its binding with the MCM complex, leading to its fall off from the chromatin (Fig. 5D). Consistent with this model, we showed that DNA damage-induced Chk1 phosphorylation at ATR sites was reduced in a manner dependent on the expression level of the MCM complex. Further, mutating the ATR phosphorylation site of Chk1 slightly increased the interaction between Chk1 and the MCM complex. Most importantly, Chk1 phosphorylated at ATR sites failed to interact with MCMs. Previously, the MCM complex was reported to be required for Chk1 phosphorylation due to its role in the activation of replication checkpoints (7, 9). Our data add a novel mechanism by which the MCM complex regulates Chk1 phosphorylation through recruiting Chk1 onto chromatin.

**REFERENCES**

1. Zhang, Y., and Hunter, T. (2014) Roles of Chk1 in cell biology and cancer therapy. Int. J. Cancer 134, 1013–1023
2. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. Genes Dev. 14, 1448–1459
3. Takai, H., Tominaga, K., Motoyama, N., Minamisawa, Y. A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., and Nakaniishi, M. (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1−/− mice. Genes Dev. 14, 1439–1447
4. Lam, M. H., Liu, Q., Elledge, S. J., and Rosen, J. M. (2004) Chk1 is haploinsufficient for multiple functions critical to tumor suppression. Cancer Cell 6, 45–59
5. Cimprich, K. A., and Cortez, D. (2008) ATR: an essential regulator of genome integrity. Nat. Rev. Mol. Cell Biol. 9, 616–627
6. Zhang, Y. W., Otterness, D. M., Chiang, G. G., Xie, W., Luo, Y. C., Mercurio, F., and Abraham, R. T. (2005) Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. Mol. Cell 19, 607–618
7. Cortez, D., Glick, G., and Elledge, S. J. (2004) Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. Proc. Natl. Acad. Sci. U.S.A. 101, 10078–10083
8. Tercero, J. A., Longhese, M. P., and Diffley, J. F. (2003) A central role for DNA replication forks in checkpoint activation and response. Mol. Cell 11, 1323–1336
9. Tsao, C. C., Geisen, C., and Abraham, R. T. (2004) Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. EMBO J. 23, 4660–4669
10. Zhang, Y. W., Brognard, J., Coughlin, C., You, Z., Dolled-Filhart, M., Aslanian, A., Manning, G., Abraham, R. T., and Hunter, T. (2009) The F box protein Fbx6 regulates Chkl stability and cellular sensitivity to replication stress. Mol. Cell 35, 442–453
11. Wang, J., Engle, S., and Zhang, Y. (2011) A new in vitro system for activating the cell cycle checkpoint. Cell Cycle 10, 500–506
12. Tsuji, T., Ficarro, S. B., and Jiang, W. (2006) Essential role of phosphorylation of MCM2 by Cdc7/Dbf4 in the initiation of DNA replication in mammalian cells. Mol. Biol. Cell 17, 4459–4472
13. Wang, J., Han, X., Feng, X., Wang, Z., and Zhang, Y. (2012) Coupling cellular localization and function of checkpoint kinase 1 (chk1) in checkpoints and cell viability. J. Biol. Chem. 287, 25501–25509
14. Méndez, J., and Stillman, B. (2000) Chromatin association of human origin recognition complex, Cdc6, and minichromosome maintenance proteins.
during the cell cycle: assembly of prereplication complexes in late mitosis. Mol. Cell. Biol. 20, 8602–8612
15. McDonald, W. H., Tabb, D. L., Sadygov, R. G., MacCoss, M. J., Venable, J., Graumann, J., Johnson, J. R., Cociorva, D., and Yates, J. R., 3rd. (2004) MS1, MS2, and SQRT-three unified, compact, and easily parsed file formats for the storage of shotgun proteomic spectra and identifications. Rapid Commun. Mass Spectrom. 18, 2162–2168
16. Eng, J. K., McCormack, A. L., and Yates, J. R., 3rd (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. 5, 976–989
17. Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., and Gygi, S. P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. J. Proteome Res. 2, 43–50
18. Tabb, D. L., McDonald, W. H., and Yates, J. R., 3rd. (2002) DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J. Proteome Res. 1, 21–26
19. Smits, V. A., Reaper, P. M., and Jackson, S. P. (2006) Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr. Biol. 16, 150–159
20. Leung-Pineda, V., Huh, J., and Piwnica-Worms, H. (2009) DDB1 targets Chk1 to the Cuhe E3 ligase complex in normal cycling cells and in cells experiencing replication stress. Cancer Res. 69, 2630–2637
21. Kumagai, A., and Dunphy, W. G. (2000) Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. Mol. Cell 6, 839–849
22. Scorah, J., Dong, M. Q., Yates, J. R., 3rd, Scott, M., Gillespie, D., and McGowan, C. H. (2008) A conserved proliferating cell nuclear antigen-interacting protein sequence in Chk1 is required for checkpoint function. J. Biol. Chem. 283, 17250–17259
23. Forsburg, S. L. (2008) The MCM helicase: linking checkpoints to the replication fork. Biochem. Soc. Trans. 36, 114–119
24. Tye, B. K., and Sawyer, S. (2000) The hexameric eukaryotic MCM helicase: building symmetry from nonidentical parts. J. Biol. Chem. 275, 34833–34836
25. Wang, J., Han, X., and Zhang, Y. (2012) Autoregulatory mechanisms of phosphorylation of checkpoint kinase 1. Cancer Res. 72, 3786–3794
26. Ibarra, A., Schwob, E., and Méndez, J. (2008) Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. Proc. Natl. Acad. Sci. U.S.A. 105, 8956–8961
27. Stracker, T. H., Usui, T., and Petrini, J. H. (2009) Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. DNA Repair (Amst.) 8, 1047–1054

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