Identification of MCM4 as a Target of the DNA Replication Block Checkpoint System*

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Inhibition of the progression of DNA replication prevents further initiation of DNA replication and allows cells to maintain arrested replication forks, but the proteins that are targets of the replication checkpoint system remain to be identified. We report here that human MCM4, a subunit of the putative DNA replication helicase, is extensively phosphorylated in HeLa cells when they are incubated in the presence of inhibitors of DNA synthesis or are exposed to UV irradiation. The data presented here indicate that the consecutive actions of ATR-CHK1 and CDK2 kinases are involved in this phosphorylation in the presence of hydroxyurea. The phosphorylation sites in MCM4 were identified using specific anti-phosphoantibodies. Based on results that showed that the DNA helicase activity of the MCM4-6-7 complex is negatively regulated by CDK2 phosphorylation, we suggest that the phosphorylation of MCM4 in the checkpoint control inhibits DNA replication, which includes blockade of DNA fork progression, through inactivation of the MCM complex.

The initiation of DNA replication in eukaryotes is triggered by activation of the MCM1 (2–7) complex, which is loaded with CDC6 and CDT1 onto replication origins to which ORCs are bound (1–3). The activation of the MCM complex requires phosphorylation of the MCM2 subunit by CDC7/DBF4 kinase and association of CDC45 with the origin region after the action of cyclin-dependent kinase. The MCM2-7 complex is the most likely candidate to act as the DNA replication helicase that catalyzes the unwinding of the DNA duplex during replication (4, 5). Although all MCM subunits possess a DNA-dependent ATPase motif in their central domain, DNA helicase activity is detected only with the MCM4-6-7 complex, which dimerizes to form a hexamer (6–9). Thus, it is possible that the MCM4-6-7 hexamer is an activated form of the MCM (2–7) hexamer, although other mechanisms leading to the activation of the MCM helicase activity have been proposed (10). MCM4 is phosphorylated in vivo at least in part by cyclin-dependent kinases, which probably leads to the inactivation of the MCM complex (11, 12). We reported previously (13) that the DNA helicase activity of the MCM4-6-7 complex is inhibited by the site-specific phosphorylation of MCM4 with CDK2-cyclin A. In Saccharomyces cerevisiae, it has been shown that cyclin-dependent kinases play a role leading to the exclusion of MCM4 from the nucleus (14). Other targets of the cyclin-dependent kinase activity that contribute to the negative regulation of DNA replication include ORC, CDC6, and CDT1 (15).

Cells normally protect the integrity of their genome from stresses such as ultraviolet light, ionizing radiation, alkylating reagents, and DNA replication blockage (16–21). Treatment with hydroxyurea (HU), which inhibits ribonucleotide reduction, not only blocks the progression of DNA replication but also activates a DNA replication checkpoint system that is required to maintain genomic integrity. In the presence of HU or methyl methanesulfonate, the initiation of DNA replication at late origins is prevented (22–24), and the arrested replication fork structure is maintained (25) by an active process that includes protein phosphorylation by the Mec1 and Rad53 kinases in S. cerevisiae. Recently, Sogo et al. (26) showed that the accumulation of single-stranded DNA and replication fork reversion occur at stalled replication forks in the absence of checkpoint control. It is plausible that these abnormal DNA structures lead to a loss of genome integrity. Mec1 with Rad3-1-1 kinases are known to be sensors of the arrested fork structure, and Rad53 is an effector kinase that is activated by its phosphorylation by Mec1. Several target proteins in the replication checkpoint pathway have been identified. Rad53 phosphorylates Dbf4 to attenuate the Cdc7/Dbf4 kinase activity (27–29), and replication protein A, a single-stranded DNA-binding protein, is phosphorylated by Mec1 in an HU-dependent manner (30). The phosphorylation of DNA polymerase α is also regulated in this system (31). However, it remains unclear whether these targets are necessary and sufficient for the checkpoint reactions (32). In higher eukaryotes, ATR (Mec1 homolog), which binds to the arrested fork structure with other sensor proteins, phosphorylates CHK1, an effector kinase, leading to its activation (33, 34). Chinese hamster ovary cells lacking CHK1 function show a progressive change in the global pattern of replication origin firing in the absence of any DNA replication (34). Target protein(s) in the replication checkpoint system remain to be identified.

In this study we identified MCM4 as a target of the replication checkpoint system. The results suggest that the consecutive actions of ATR-CHK1 and CDK2 are required for the
phosphorylation of MCM4. The phosphorylation of MCM4 should inhibit DNA replication through the inactivation of the MCM complex.

EXPERIMENTAL PROCEDURES

Reagents—Caffeine, hydroxyurea, and 2-aminopurine were purchased from Sigma, and Go6976 was from Calbiochem. Aphidicolin was purchased from Wake Pure Chemical. Antibodies against CHK1 were purchased from Santa Cruz Biotechnology, and CDK2-Thr-160 phosphorylation antibodies were from Cell Signaling. Anti-MCM4 antibodies were obtained as reported (35). Histidine-tagged human CHK1 and CHK2 proteins were expressed in insect cells using a baculovirus expression system (36, 37) and purified by nickel-nitriilotriacetic acid column chromatography according to the manufacturer’s protocol (Qiagen). Human MCM4 and -6 genes were cloned into a pAcUW31 vector (7), and the MCM7 gene was cloned into a pVL1392 vector for baculovirus expression. An MCM4-6-7 complex containing histidine-tagged MCM4 was purified from the viral infected High5 cells by nickel-nitriilotriacetic acid column chromatography and then MonoQ column chromatography. The human MCM4-6-7 complex (50 ng) was incubated with increasing levels of CHK1 (120 and 400 ng) in 50 mM Hepes-NaOH, pH 8, 10 mM MgCl2, 2.5 mM EGTA, 1 mM dithiothreitol, and 100 µM [γ-32P]ATP or incubated with CDK2-cyclin A (20, 60, and 200 ng) in 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 10 mM MgCl2, 0.1% Triton X-100, and 100 µM [γ-32P]ATP. The reaction mixtures were incubated for 1 h at 37°C, and the products were analyzed by electrophoresis on 10% polyacrylamide gel containing SDS.

Purification of MCM4-6-7 Complex from HeLa Cells—HeLa cells were cultured in the presence or absence of HU (2 mM) for 24 h. The harvested cells (~1 × 10^6 cells) were washed with phosphate-buffered saline and then stored at ~80°C. After thawing, the cells were suspended in modified CSK buffer containing phosphatase inhibitors (10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 50 mM sodium fluoride) in the absence of Triton X-100 and kept at 0°C for 15 min. The cell precipitate recovered after centrifugation was then suspended in CSK buffer containing 0.1% Triton X-100, 0.4 M NaCl, and phosphatase inhibitors and kept at 0°C for 15 min. The recovered supernatant fraction contained about half the chromatin-bound MCM proteins. After centrifugation, the solubilized proteins were loaded onto a histone H3/H4 column (2 ml) that had been equilibrated with a buffer containing 0.3 M NaCl (6). The column was extensively washed with the same buffer, and proteins were eluted with a linear gradient of 0.3–2 M NaCl. The fractions containing MCM4, -6,
and -7 proteins that were eluted with 0.6–0.9 M NaCl were pooled and concentrated with Centricon 30 (Amicon) in the presence of phosphatase inhibitors (Phosphatase Inhibitor Mixture 1 plus 2 from Sigma) and protease inhibitors. During the concentration, the solution was diluted to 0.15 M NaCl. The diluted and concentrated sample was further purified by glycerol gradient centrifugation. Fractions 3–6 of a total of 15 fractions were pooled and concentrated to 20 μl with Microcon (Amicon) after the addition of protease inhibitors. The concentrated sample, which contained ~35 μg/ml proteins, was used for measuring the displacement of 17-mer oligonucleotide annealed to M13 single-stranded DNA by DNA helicase activity (6).

Another procedure was employed to obtain total cell extracts in the absence of phosphatase inhibitors. HeLa cells were washed with hypotonic buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, and 1 mM dithiothreitol) and then homogenized with Dounce homogenizer (B pestle). Proteins extracted with 0.4 M NaCl in the same solution were loaded onto a histone H3/H4 column equilibrated with 0.3 M NaCl, and bound proteins were eluted by the linear gradient from 0.3 to 2 M NaCl. Fractions containing MCM4, -6, and -7 proteins were pooled and further purified by glycerol gradient centrifugation as described above, except that fractions obtained after glycerol gradient centrifugation were used without concentration for measuring DNA helicase activity.

UV Irradiation of HeLa Cells—HeLa cells that were logarithmically growing in dishes (100 mm in diameter) were exposed to UV irradiation (100 J/m2) from a germicidal lamp (Toshiba GL15) at 2.5 J/m2 in the absence of growth medium. Fresh growth medium (Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum) was added to the cells, and they were cultured at 37 °C for the indicated periods. Cells harvested after incubation with trypsin/EDTA solution were washed with phosphate-buffered saline and stored at ~80 °C before fractionation of the cells.

**RESULTS**

Phosphorylation of MCM4 in the Presence of HU—Logarithmically growing HeLa cells were cultured in the presence of HU for different periods (0, 4, 8, 16, and 24 h). After lysis, cells were fractionated into Triton-soluble (S1 and S2) and Triton-insoluble chromatin-bound fractions (P). The proteins in these fractions were reacted with anti-MCM4 antibodies (Fig. 1A, top panel). MCM4 was detected in both the soluble (S1) and the insoluble chromatin-bound (P) fractions isolated from cells cultured in the absence of HU. A portion of the MCM4 with slightly retarded mobility was detected in the P fraction. During incubation in the presence of HU, the level of MCM4 in the P fraction with retarded mobility gradually increased with maximal retardation observed after 16 h. In contrast, the mobility of the MCM4 in the S1 fraction did not change during the 24 h of incubation in the presence of HU. The alteration in the mobility of MCM4 appeared to be due to its phosphorylation because after λ-phosphatase treatment it migrated to a position identical to that observed for the MCM4 isolated from the S1 fraction (Fig. 1B). The phosphorylation of CHK1 was examined during incubation of cells with HU (Fig. 1A, middle panel).

Retention of its mobility, which is due to phosphorylation by ATR (34), was detected over a time course similar to that observed for the hyperphosphorylation of MCM4. It remains to be determined why the total amount of CHK1 protein decreases after 24 h of incubation with HU.

Checkpoint-dependent Phosphorylation of MCM4—To address the question of whether the observed phosphorylation of...
MCM4 is due to the action of the DNA replication checkpoint system, caffeine, an inhibitor of ATR/ATM, which plays a central role in the checkpoint system, was added to the growth medium containing HU. As shown in Fig. 2 (top panel), this addition markedly reduced the hyperphosphorylation of MCM4 isolated from the P fraction. The reduced level of hyperphosphorylated MCM4 in the presence of caffeine appears to be associated with the dephosphorylation of CHK1 protein, which is evident from the mobility shift of retarded CHK1 to the basal position (Fig. 2, middle panel). To clarify the role played by CHK1 kinase in the hyperphosphorylation of MCM4, G66976, an inhibitor of CHK1 kinase (38) but not CDK2 kinase or CHK2 kinase activity (Fig. 3, A and B), was added to the medium containing HU. The drug inhibited the hyperphosphorylation in a dose-dependent manner (Fig. 3C, top panel). At a concentration of 600 nM, hyperphosphorylation of MCM4 was largely prevented, whereas phosphorylation of CHK1 protein itself was unaffected by G66976 (Fig. 3C, middle panel), indicating that this drug does not affect ATR kinase activity. These findings suggest that an ATR-CHK1 pathway is involved in the hyperphosphorylation of MCM4.

Involvement of Cyclin-dependent Kinase in MCM4 Phosphorylation—In the presence of HU, ATR kinase phosphorylates CHK1 kinase, an effector kinase in the checkpoint system, to activate it in HeLa cells (Fig. 1A) (34). To identify the kinase responsible for MCM4 hyperphosphorylation in the presence of HU, we first phosphorylated the human MCM4-6-7 complex with a human CHK1 kinase preparation in vitro. Both MCM4 and -6 proteins in the complex appeared to be phosphorylated (Fig. 4A, right panel), but phosphorylation with CHK1 did not lead to the generation of highly phosphorylated forms of MCM4 (Fig. 4B, 1st column on the left panel). In contrast, MCM4 products with retarded mobility were generated by phosphorylation with CDK2-cyclin A (Fig. 4, A, left panel, and B, 1st column on the left panel). We reported previously (39) that mouse MCM4 in an MCM4-6-7 complex was extensively phosphorylated with CDK2-cyclin A and that this phosphorylation inhibited the DNA helicase activity of the complex. Phosphorylation in this system of mutant mouse MCM4-6-7 complexes, where amino acids of MCM4 were altered in a site-specific manner, indicated that six sites (Ser-3, Thr-7, Thr-19, Ser-32, Ser-53, and Thr-109) in the amino-terminal region of mouse MCM4 were required for the phosphorylation of MCM4 (13). Specific phosphoantibodies against three (Ser-32, Ser-54, and Thr-110) of these sites in human MCM4 were raised, and they were examined for binding to the phosphorylated MCM4 (Fig. 4B). They interacted with the MCM4-phosphorylated product formed with CDK2-cyclin A but not with the phosphorylated MCM4 product formed with CHK1 (Fig. 4B, 2nd to 4th columns on the left panel). Next, we examined the interaction of these phosphoantibodies with MCM4-hyperphosphorylated products produced in the presence of HU (Fig. 4B, 2nd to 4th columns on the right panel). These phosphoantibodies recognized the hyperphosphorylated MCM4 products that were isolated from the chromatin fractions (S4 and P') of HU-treated HeLa cells. Quantification of the level of binding of these phosphoantibodies indicates that phosphorylation of MCM4 in the chromatin fractions increased by 2.5-6-fold in the presence of HU. These results strongly suggest that cyclin-dependent kinase activity is involved directly in the HU-induced hyperphosphorylation of MCM4.

To gain more insight on the mechanism by which MCM4 is hyperphosphorylated by a cyclin-dependent kinase, we monitored the activation of CDK2 by examining its phosphorylation at Thr-160 (40) during the incubation of HeLa cells with HU (Fig. 1A, bottom panel). Phosphorylation of Thr-160 increased 2-3-fold after 16 and 24 h of the incubation with HU. The addition of caffeine (Fig. 2, bottom panel) or G66976 (Fig. 3C, bottom panel) decreased the level of phosphorylated CDK2 produced in the presence of HU. These results indicate that the activation of CDK2 kinase activity correlates with the increased level of MCM4 hyperphosphorylation induced by HU.

Functional Significance of Phosphorylation of MCM4—To understand the functional significance of phosphorylation of MCM4, DNA helicase activity was compared between MCM4-6-7 complexes prepared from HU-treated and non-treated HeLa cells. After extraction of a large portion of the soluble forms of the MCM complex, approximately half of the chromatin-bound MCM complex was recovered by extraction of the remaining insoluble fraction with a buffer containing 0.4 M NaCl (data not shown). The MCM4-6-7 complex was purified from the 0.4 M NaCl-soluble fraction of HU-treated HeLa cells and non-treated cells in the presence of phosphatase inhibitors, and it was also purified from total cell extracts of non-treated cells in the absence of phosphatase inhibitors as described under “Experimental Procedures.” As shown in Fig. 5A, these three

![Diagram](http://www.jbc.org/)

**Fig. 4.** Phosphorylation of MCM4 by cyclin-dependent kinase. A, human MCM4-6-7 complex (50 ng) was incubated with increasing amounts of CDK2-cyclin A (left panel) or CHK1 (right panel). Phosphorylated proteins were analyzed by electrophoresis on 10% polyacrylamide gel, followed by autoradiography. The maximal level of 32P incorporation into MCM4 by CDK2 was 2.3 mol/mol of MCM4 and that of 32P incorporated into MCM4 + MCM6 by CHK1 was 4 mol/mol of MCM4 + MCM6. B, left panel, human MCM4-6-7 complex was phosphorylated by CHK1 or CDK2-cyclin A in vitro, and products were analyzed by SDS-PAGE. Proteins were analyzed by Western blotting using MCM4 antibodies (1st column) and phosphoantibodies against amino acids Ser-32, Ser-54, and Thr-110 of MCM4 (2nd to 4th columns), as indicated. Right panel, the soluble (S1) and chromatin-bound fractions (S4 and P') prepared from HeLa cells treated with HU were analyzed by Western blotting using MCM4 and the anti-phosphoantibodies. Bars at the left of gels indicate the normal position of unphosphorylated MCM4.
MCM4-6-7 complexes exhibited similar protein compositions and concentration, although only a slight amount of MCM2 was detectable in the complexes prepared from HU-treated cells in the presence of phosphatase inhibitors and from non-treated cells in the absence of phosphatase inhibitors. Two MCM4 bands that migrated closely on a gel were detected from the complex prepared from non-treated cells in the absence or presence of phosphatase inhibitors, although the ratio of these two bands varied between the two complexes. Only one MCM4 band with retarded mobility was detected in the MCM4-6-7 complex prepared from HU-treated cells in the presence of phosphatase inhibitors. It is probable that these two MCM4 bands differed in terms of the level of phosphorylation as described below. The DNA helicase activity of the purified MCM4-6-7 complexes was examined (Fig. 5, C and D). The MCM4-6-7 complex prepared from non-treated HeLa cells in the absence of phosphatase inhibitors exhibited the activity in a dose-dependent manner. However, the complex prepared from non-treated and HU-treated cells in the presence of phosphatase inhibitors showed a reduced level of the helicase activity. These three preparations of MCM4-6-7 complexes were examined for the phosphorylation status of MCM4 by using anti-phosphoantibodies (Fig. 5B). In addition to the three antibodies (Ser(P)-32, Ser(P)-54, and Thr(P)-110), two other antibodies (Thr(P)-7 and Thr(P)-19) were used for the detection of phosphorylation of MCM4 (Fig. 5B). As shown in Fig. 5B, panel at the far right, almost identical amounts of MCM4 were loaded onto the gels. It appears that partial dephosphorylation of MCM4 occurred during the purification of the MCM4-6-7 complex even in the presence of phosphatase inhibitors, because highly phosphorylated forms of MCM4 with retarded mobility were not evident in the purified complex prepared from HU-treated cells. However, immunoblotting studies using these five phosphoantibodies show that MCM4 in the MCM4-6-7 complex prepared from HU-treated cells and non-treated cells in the presence of phosphatase inhibitors is more phosphorylated than MCM4 in the complex prepared from non-treated cells in the absence of phosphatase inhibitors. These results suggest that the DNA helicase activity of MCM4-6-7 complex is negatively regulated by phosphorylation of MCM4 at these five sites. This notion is supported by the findings showing that DNA helicase activity of mouse MCM4-6-7 complex is inhibited by the site-specific phosphorylation (Ser-3, Thr-7, Thr-19, Ser-32, Ser-53, and Thr-109) of MCM4 with CDK2-cyclin A (13). These results also indicate that phosphorylation of MCM4 at these sites occurs even in the absence of HU.

Aphecidolcin- and UV-dependent Phosphorylation of MCM4—To examine whether phosphorylation of MCM4 is stimulated by other DNA-damaging reagents, HeLa cells were incubated in the presence of aphecidolcin, which is an inhibitor of DNA polymerases including DNA polymerase α, or they were exposed to UV irradiation (Fig. 6). In the presence of aphidicolin, phosphorylation of both MCM4 and CHK1 proteins was stimulated (Fig. 6A). Addition of 2-aminopurine, which is another inhibitor of ATR/ATM kinase (41), inhibited the HU-dependent phosphorylation of MCM4 and CHK1 proteins. We believe that bands detected in the P fractions when anti-CHK1 antibodies were used as a probe are not CHK1 proteins. The HU-dependent phosphorylation of MCM4 and CHK1 proteins was partially prevented by the presence of caffeine (data not shown). During incubation after UV irradiation, phosphorylation of both MCM4 and CHK1 proteins was gradually stimulated (Fig.
pathway somehow activates CDK2 activity in the presence of HU, the results presented here suggest that the ATR-CHK1 pathway activates CDK2 activity leading to the phosphorylation of MCM4.

Regarding the role of cyclin-dependent kinase in the DNA replication checkpoint system, Pellicioli et al. (31) have reported that the phosphorylation of the B subunit of DNA polymerase α in 

S. cerevisiae

is negatively regulated by the presence of HU. They suggested that the kinase activity of Cdk1, which phosphorylates the B subunit, is inhibited in a Rad53-dependent manner. It has been reported (43) that the Srs DNA helicase, which may be involved in the processing of stalled replication forks, is phosphorylated in the presence of HU in a Rad53-dependent reaction. In contrast to the phosphorylation of the B subunit of DNA polymerase α, phosphorylation of the Srs helicase required the action of the cyclin-dependent kinase Cdk1. These results suggest that two opposing reactions, one leading to the activation and one resulting in the inhibition of Cdk1 activity, are involved in the replication checkpoint system. An important role of Cdk2 in other checkpoint systems has been reported; the G1 checkpoint in hematopoietic cells leads to the activation of Cdk2 (44).

The MCM2-7 proteins play a central role in the initiation and elongation of DNA replication. It is likely that they act as a DNA-unwinding enzyme in DNA replication. We reported that the DNA helicase activity of the human and mouse MCM4-6-7 complex, a sub-complex of the MCM2-7 heterohexamer, is inhibited by the phosphorylation by Cdk2-cyclin A (13, 39). We identified six sites, including Ser-32, Ser-53, and Thr-109, in the amino-terminal region of mouse MCM4 that are required for the phosphorylation with CDK2-cyclin A. Changes of these sites to alanine made the mutant MCM4-6-7 complex resistant to inhibition of the DNA helicase activity by Cdk2-cyclin A, indicating that the site-specific phosphorylation of MCM4 with Cdk2-cyclin A causes the inhibition of DNA helicase activity (13). The finding that phosphorylation of MCM4 at these sites was stimulated in the presence of HU suggests the notion that the replication checkpoint-dependent phosphorylation of MCM4 results in the inhibition of DNA replication through the inactivation of the MCM complex. We presented evidence showing that the MCM4-6-7 complex prepared from HU-treated HeLa cells exhibited a reduced level of DNA helicase activity, but the MCM4-6-7 complex prepared from non-treated HeLa cells also exhibited a reduced level of the activity. Comparison of the phosphorylation status among these two MCM complexes and the MCM4-6-7 complex, which is more active as a DNA helicase, suggests that the phosphorylation of MCM4 at Thr-7, Thr-19, Ser-32, Ser-54, and Thr-110 is involved in lowering the helicase activity. These results suggest that the DNA helicase activity of the MCM4-6-7 complex prepared from the chromatin-bound fraction of non-treated HeLa cells is nega-

**Fig. 6.** Proposed transduction pathway leading to the hyper-phosphorylation of MCM4 and the targets of inhibitors used in this study. We speculate that in the presence of HU the ATR-CHK1 pathway activates CDK2 activity leading to the phosphorylation of MCM4.

**DisCUSSION**

We report here that MCM4, a subunit of the putative repli-cative DNA helicase, is phosphorylated in the presence of inhibitors of DNA synthesis or after exposure to UV irradiation. ATR and the downstream kinase CHK1 are involved in the activation of a DNA replication checkpoint in the presence of HU in HeLa cells (34). Consistent with this observation, our results suggest that an ATR-CHK1 pathway is mainly responsible for the hyperphosphorylation of MCM4 in the presence of HU. It should be noted, however, that neither caffeine nor G66976 completely block the MCM4 phosphorylation, suggesting that another pathway is also involved in the phosphorylation. Although it is possible that CHK1 phosphorylates MCM4 directly in the presence of HU, the results presented here suggest that a cyclin-dependent kinase is mainly responsible for the observed phosphorylation. We propose that the ATR-CHK1 pathway somehow activates CDK2 activity in the presence of HU to hyperphosphorylate MCM4 (Fig. 7). In agreement with this notion, it has been reported that an assembly factor of CDK-activating kinase, which activates CDK2 by phosphorylating it at Thr-160, interacts with MCM7 (42).

**Fig. 7.** Phosphorylation of MCM4 in the presence of aphidicolin or after UV irradiation. A, logarithmically growing HeLa cells were cultured in the presence or absence of aphidicolin (15 μM) and 2-aminopurine (2-AP) (5 or 10 mM) for 24 h as indicated. The cells were fractionated into soluble S (S1 plus S2) and insoluble P fractions, and proteins in these fractions were separated by SDS-gel electrophoresis for analysis of MCM4 and CHK1 proteins. B, HeLa cells were exposed to UV irradiation (100 J/m²) and then cultured for indicated periods in the absence or the presence of caffeine (5 mM). Soluble S and insoluble P fractions were analyzed for MCM4 and CHK1 proteins.
tively regulated by phosphorylation of MCM4 with cyclin-dependent kinase, and the treatment of HeLa cells with HU stimulates the phosphorylation of MCM4 with the kinase.

Because MCM is the most likely candidate for the replicative helicase that is responsible for the replication fork movement, it is possible that the phosphorylation of MCM4 explains phenotypic events observed upon activation of the replication checkpoint control system in S. cerevisiae and Chinese hamster ovary cells. Inhibition of MCM helicase activity by CDK2 may be involved in preventing the accumulation of single-stranded DNA at stalled replication forks that was detected in S. cerevisiae (26) and preventing the initiation of DNA replication in the presence of checkpoint control (22, 23, 34). Thus, we propose that MCM4 is one of the crucial targets of the DNA replication checkpoint system.

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