Phosphorylation of MCM4 by Cdc7 Kinase Facilitates Its Interaction with Cdc45 on the Chromatin*§

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Cdc7 kinase, conserved from yeasts to human, plays important roles in DNA replication. However, the mechanisms by which it stimulates initiation of DNA replication remain largely unclear. We have analyzed phosphorylation of MCM subunits during cell cycle by examining mobility shift on SDS-PAGE. MCM4 on the chromatin undergoes specific phosphorylation during S phase. Cdc7 phosphorylates MCM4 in the MCM complexes as well as the MCM4 N-terminal polypeptide. Experiments with phospho-amino acid-specific antibodies indicate that the S phase-specific mobility shift is due to the phosphorylation at specific N-terminal (S/T)(S/T)P residues of the MCM4 protein. These specific phosphorylation events are not observed in mouse ES cells deficient in Cdc7 or are reduced in the cells treated with siRNA specific to Cdc7, suggesting that they are mediated by Cdc7 kinase. The N-terminal phosphorylation of MCM4 stimulates association of Cdc45 with the chromatin, suggesting that it may be an important phosphorylation event by Cdc7 for activation of replication origins. Deletion of the N-terminal non-conserved 150 amino acids of MCM4 results in growth inhibition, and addition of amino acids carrying putative Cdc7 target sequences partially restores the growth. Furthermore, combination of MCM4 N-terminal deletion with alanine substitution and deletion of the N-terminal segments of MCM2 and MCM6, respectively, which contain clusters of serine/threonine and are also likely targets of Cdc7, led to an apparent nonviable phenotype. These results are consistent with the notion that the N-terminal phosphorylation of MCM2, MCM4, and MCM6 may play functionally redundant but essential roles in initiation of DNA replication.

DNA replication proceeds through series of staged reactions involving various protein-DNA and protein-protein interactions on template DNA. In eukaryotes, origin recognition complexes are believed to play central roles in recognition of replication origins and to function as landing pads for other essential replication factors including MCM (minichromosome maintenance proteins) (1, 2). The initiation of DNA replication is under strict regulation of G1 cell cycle signals, which are activated or suppressed by extracellular growth or differentiation signals, respectively (3). The G1 cell cycle signals regulate Cdk-cyclins and ultimately activate E2F, leading to activation of various components of replication machinery as well as protein kinases (4). Cdk2-cyclinE and Cdc7-Dbf4 kinase are among those activated by the G1 signals and are known to play critical roles in activation of DNA replication origins (5–11).

The critical targets of these kinases in initiation of DNA replication are not well understood, but subunits of the MCM complexes, which may play a critical role in origin activation as well as in the elongation stage of DNA replication (12–14), are likely to be among the important substrates of these kinases (9, 15). Among them, MCM2 has been shown to be phosphorylated by Cdc7 kinase both in vivo and in vitro in yeasts as well as in Xenopus egg extracts and mammalian cells (16–24). In fission yeast, Hsk1 kinase, the homologue of budding yeast Cdc7 kinase, was shown to phosphorylate MCM4 in the MCM2-MCM4-MCM6-MCM7 complex (25). However, precise Cdc7-mediated phosphorylation sites on the MCM subunits are not known, except for a recent report on the N-terminal segment of MCM2 (17, 20), nor is the significance of these phosphorylation events known. Only recently, a potential role of phosphorylation of MCM2 N-terminal segment was reported in human cells (48).

In this report, we have analyzed phosphorylation of the MCM4 protein in vivo and discovered that Cdc7 is required for this phosphorylation. We have further identified phosphorylation sites on MCM4, which are mediated by Cdc7, and have shown that Cdc7-mediated phosphorylation may play important roles in loading of Cdc45 onto the chromatin. We also show the data suggesting that the phosphorylation of the N-terminal serine/threonine clusters of MCM subunits by Cdc7 may play important but redundant roles in initiation of DNA replication.

EXPERIMENTAL PROCEDURES

Cell Synchronization and Preparation of Cell Lysates—HeLa cells were arrested at the G1/S boundary by two successive incubation in the medium containing 2.5 mM thymidine (24 h...
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for each) with an interval of 12 h of growth without thymidine. Cells were then released into medium without thymidine and were harvested at each time point. Cells were also arrested at early S with 0.5 mm mimosine for 24 h, 2 mm HU2 for 12 h, respectively, followed by fractionation into Triton-soluble and -insoluble (chromatin-enriched) fractions, as described previously (26, 27). The whole cell extract was the soluble supernatant of the cells sonicated in CSK buffer (26) containing 0.1% Triton X-100. Each fraction was applied on 7.5% SDS-PAGE and MCM2 protein was detected by Western blotting. Synchronization of cell cycle was monitored by flow cytometry analyses of the cells stained with propidium iodine.

Small Interfering RNA (siRNA) and Transfection—Transfection of siRNA, purchased from Japan Bio Services (Saitama, Japan), was conducted by using Oligofectamine (Invitrogen). The siRNA for Cdc7 were Cdc7–1 (27), Cdc7–D (28), or Cdc7–nc (guaaccccuuagcuggcauTT/augccagcuaagggguuacTT).

Construction of Expression Plasmids of Mutant MCM Proteins—The mouse wild-type Mcm4 cDNA was used as a template for PCR mutagenesis to mutate each conserved serine and threonine residues to alanine or glutamic acid. The 6AA or 6EE mutant of MCM4 represents those MCM mutants in which serine and threonine residues of (S/T)(S/T)P at the positions 2–4, 6–8, 30–32, 52–54, 69–71, and 86–88 were replaced with alanine (Ala) or glutamic acid (Glu). Alanine or glutamine mutants of fission yeast MCM2(Cdc19) and MCM3(Cdc7) were from Dr. Susan Forsburg and that against MCM6(Mis5) protein was from Dr. Hisao Masukata. Anti-CDt1 antibody was from Dr. Hideo Nishitani.

Yeast Strains and Plasmids—Methods for genetic and biochemical analyses of fission yeast have been described previously (30). The following strains were used for this study: NI740 (h+ ade6-M210 ura4-D18 leu1–32, NI284 (h+ ade6-M216 ura4-D18 leu1–32 cdc21-M68), CHP429 (h+ ade6-M216 ura4 leu1 his7), MS190 (h+ ura4 leu1 his7 cdc19-1P1), MS210 (h+ ade6-M216 ura4 leu1 his7 Δcdc21::[Pnmt-cdc21::his7+]), MS211 (h+ ade6 ura4 leu1 his7 Δcdc21::[Pnmt-D67cdc21::his7+]), MS213 (h+ ade6 ura4 leu1 his7 Δcdc21::[Pnmt-D130cdc21::his7+]), MS240 (h+ ade6-M210 ura4 leu1 his7 Δ47mis5::kan), MS242 (h+ ade6-M210 ura4 leu1 his7 mis5::kan), and Goa1-HA (h+ goa1-HA3 ura4-D14 leu1–32 ade6-M26) and Goa1-HA hsk1–89 (h+ goa1-HA3 leu1–32 ade6-M210 ura4-D18 hsk1–89::ura4+).

RESULTS

Cell Cycle-dependent Phosphorylation of Human MCM4 Protein—MCM4 protein was previously reported to be a phosphoprotein (31, 32). Therefore, we have examined the phosphorylation of MCM4 protein during the cell cycle. HeLa cells

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2 The abbreviations used are: HU, hydroxyurea; siRNA, small interfering RNA; GST, glutathione S-transferase; PCNA, proliferating cell nuclear antigen; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; tg, transgene.

3 K. Tamai, unpublished data.
were synchronized by release from double thymidine block, which arrests the cells at the G1-S boundary. The synchronous progression of the cell cycle was confirmed by FACS analyses of the DNA stained with propidium iodide (Fig. 1A). Immediately after release, the cells entered S phase, completing it in 8 h. They underwent mitosis at 10–14 h and reentered the next S phase at 18–24 h. We have prepared Triton-soluble and -insoluble extracts from the cells at each stage and analyzed the profiles of various proteins by Western blotting. The former contains the cytoplasmic and nuclear soluble proteins and the latter chromatin-associated or insoluble proteins.

The PCNA protein, known to associate with replication forks (33), was detected in the Triton-insoluble fractions only during the S phase, until 6 h after release and later on (Fig. 1B). Cdt1 was detected in the Triton-soluble fractions mainly during G1 phase at 12–18 h after release (34). α-Tubulin, a marker for the cytoplasmic protein, was constitutively detected at a constant level in the Triton-soluble fractions, whereas the LaminB protein was constitutively detected in the chromatin-enriched fractions, verifying the fractionation procedure. A portion of MCM4 was detected in the insoluble fractions at the time of release but its level decreased as the S phase progressed. Other MCM proteins also behaved in a similar manner; about a half-population associates with chromatin during G1, and dissociates from the chromatin in late S to G2/M phase, whereas the remainder is detected in the Triton-soluble fraction throughout the cell cycle. This is consistent with the previous results in the Xenopus egg extracts and mammalian cells that MCM is released from the chromatin during the S phase (31, 32). MCM4 displays characteristic mobility shift on SDS-PAGE during the cell cycle. At the time 0–6 h after the release, slow migrating bands were detected in the insoluble fractions and they appeared again at 16–20 h after the release. This mobility shift was eliminated by prior treatment with phosphatase (data not shown), indicating that it is caused by phosphorylation. In the Triton-soluble fractions, highly mobility-shifted and slow migrating forms accumulated at late S through M phase (6–14 h after release). This phosphorylation was previously reported and was shown to be caused by Cdc2 kinase, as a part of the strategies to ensure the inhibition of the rereplication (31).

**In Vitro Phosphorylation of MCM4**—We have examined whether MCM4 is phosphorylated by Cdc7 in vitro. We have used the mouse MCM2-MCM4-MCM6-MCM7 complex as a substrate for the in vitro phosphorylation reactions. As reported previously, MCM2 in the MCM2-MCM4-MCM6-MCM7 was efficiently phosphorylated by Cdc7, and its mobility on SDS-PAGE shifted downward (16, 17, 19, 20; Fig. 2A). We also observed phosphorylation of MCM4 (as well as MCM6 and to a lower extent) in vitro by Cdc7. This phosphorylation caused the mobility shift of MCM4 (Fig. 2A), similar to the one observed in mammalian cells. The level of MCM4 (and MCM6) phosphorylation in the MCM2-MCM4-MCM6-MCM7 complex is lower, compared with that of MCM2 protein (~4 molecules of ATP incorporated at max on an average per molecule of MCM2 or MCM4 + MCM6, respectively; see supplemental Fig. S1). This may be due to lack of other factors, such as MCM10, which may enhance the phosphorylation reaction by Cdc7 kinase (25).

The N-terminal region of MCM4 contains the clusters of serine/threonine residues (see Fig. 7A). Some of these are the targets of Cdk, since they are present as a part of the S/T/PX motif. In vitro and in vivo phosphorylation of these serine/threonine residues by Cdk has been in fact demonstrated (31, 35). We noticed the repeated presence of (S/T)(S/T)P sequences in the N-terminal segments of MCM4 protein across the species and speculated that they may be phosphorylated by Cdc7. Therefore, we have generated mutant MCM4 in which these serine/threonine residues have been replaced by alanine. The “6AA” mutant carries alanine substitutions at the six (S/T)(S/T)P sequences at positions 2–4, 6–8, 30–32, 52–54, 69–71, and 86–88 of mouse MCM4 protein. MCM4(6AA) was expressed as a MCM2-MCM4-MCM6-MCM7 complex in insect cells. The mutation did not affect the complex formation, and the complex could be purified. Although Cdc7 could phosphorylate the MCM2 protein in the MCM2-MCM4(6AA)-MCM6-MCM7 complex, the mobility shift by the phosphorylation of MCM4 was largely eliminated, as indicated by the loss of the labeled and mobility-shifted form of MCM4 (Fig. 2A, top and bottom panels, lanes 1–3 and 6–8). Interestingly, the 6EE mutant form of MCM4, in which the same sets of serine/threonine residues were replaced with glutamic acid to generate the mutant protein mimicking the phosphorylated state, migrated anomalously on SDS-PAGE, similar to the phosphorylated forms of MCM4 (Fig. 2A, lanes 11–15).

Next, the N-terminal 198-amino acid polypeptide of MCM4 was used as a substrate for kinase reaction by Cdc2-CyclinB and Cdc7-ASK in vitro. Both Cdc7 and Cdc2 phosphorylate the N-terminal polypeptide (Fig. 2B, lanes 1 and 5). The level of phosphorylation was reduced with the 6AA mutant form of the same polypeptide (compare lanes 9 and 10 in Fig. 2B), suggesting that these (S/T)(S/T)P sequences are targets of phosphorylation by Cdk and Cdc7. However, a significant level of phosphorylation by Cdc7 was still observed with the 6AA mutant (Fig. 2B, lane 2), indicating that the other residues within the N-terminal region of MCM4 (see Fig. 7A) can be phosphorylated by Cdc7. The level of phosphorylation significantly increased in the presence of both Cdc7 and Cdc2 kinases (Fig. 2B, compare lanes 1, 5, and 9), suggesting the concerted action of the two kinases. Similar stimulatory effect of Cdk on phosphorylation of MCM2 was previously reported (17, 19, 20). However, the efficacy of MCM4 phosphorylation in the MCM4N polypeptide was significantly lower than that of MCM4 in the MCM2-MCM4-MCM6-MCM7 complex (~0.2 molecule of ATP incorporated at maximum per molecule of MCM4), suggesting that the MCM4 segment outside the N-terminal tail and/or other MCM subunits facilitate the substrate recognition by Cdc7 kinase. These results indicate that the N-terminal segment of MCM4 contains multiple Cdc7-mediated phosphorylation sites, some of which are stimulated by prior phosphorylation by Cdk.

**The N-terminal Segment of MCM4 Is Phosphorylated by Cdc7 in Vivo**—We then generated an antibody that specifically recognizes the phosphorylated forms of MCM4 at one of the above (S/T)(S/T)P residues (serine 6 and threonine 7). We have designed the phosphopeptide, which carries a phosphoserine and a phosphothreonine at the 6th and 7th position, respec-
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A

B

Triton-soluble

| Triton-insoluble (chromatin-enriched) |
|--------------------------------------|
| S | G2/M | G1 | S | S | G2/M | G1 | S | S |
|---|------|----|---|---|------|----|---|---|
| AS | 0 | 3 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 24 |

MCM4

MCM2

MCM3

MCM5

MCM4/S6T7

MCM4/T7

Cdc45

Cdc6

Cdc7

Cdt1

RPA p34

PCNA

α-tubulin

laminB
tively, as well as the one which carries only a phosphothreonine at the 7th position. The S6T7 antibody strongly reacted with MCM4N protein, when it was incubated with Cdc2-CyclinB and Cdc7 kinases (Fig. 2C, lane 3). The Western blotting of the cell cycle synchronized extract showed that the S6T7 antibody detected the mobility-shifted form of MCM4 in the Triton-insoluble fraction at 0–6 h and 18–24 h after the release from double thymidine block, suggesting that S6T7 is indeed phosphorylated by Cdc7, although the mobility shift is largely eliminated. The bands indicated by open stars represent the positions of MCM6 protein, which are phosphorylated by Cdc7, albeit to a limited extent. MCM7 is not phosphorylated by Cdc7 (see also the supplemental material). B, the wild-type (lanes 1, 5, and 9) and 6AA mutant version (lanes 2, 6, and 10) of GST-fused MCM4N protein (amino acid residues 1–198; 4 pmol) or control GST protein (lanes 3, 7, and 11; 4 pmol) was phosphorylated in vitro by the kinases indicated (Cdc7-ASK, 0.6 pmol; Cdc2-CyclinB, 0.1 pmol). Lanes 4, 8, and 12 represent reactions without a substrate. First and third panels, autoradiogram; second and fourth panels, silver-stained gel. The incorporation of ATP into the MCM4N substrates (wild-type or 6AA), estimated from radioactivity of the dried gel containing the protein band measured by a scintillation counter, was as follows (in pmol); lane 1, 0.31; lane 2, 0.27; lane 3, 0.23; lane 4, 0.06; lane 5, 0.89; and lane 6, 0.39. C, the in vitro kinase reaction was conducted as described for B except that radioactive ATP was not included. The reaction mixture was analyzed by Western blotting using the MCM4 S6T7 antibody. The reaction mixtures were analyzed on 7.5% SDS-PAGE (A) or 4–20% gradient SDS-PAGE (B and C).

FIGURE 2. In vitro phosphorylation of MCM with Cdc7-ASK. A, MCM2-MCM4-MCM6-MCM7 (2 pmol) containing the wild-type (WT) MCM4 (lanes 1–5), 6AA mutant form of MCM4 (lanes 6–10), or 6EE mutant form of MCM4 (lanes 11–15) was phosphorylated in vitro by purified Cdc7-ASK (0.6 pmol). The reaction mixtures were split into halves and run on two 7.5% (59:1) SDS-PAGE. One was for silver staining and autoradiogram, and the other was for Western blotting. Upper, silver-stained gel; middle, autoradiogram; lower, Western blotting with anti-MCM4 antibody. The bands indicated by filled stars are the positions of non-shifted MCM4 proteins (wild-type and mutants). Note that the migration of MCM4 6EE is retarded due to amino acid substitutions. MCM4 6AA is still phosphorylated by Cdc7, although the mobility shift is largely eliminated. The bands indicated by open stars represent the positions of MCM6 protein, which are phosphorylated by Cdc7, albeit to a limited extent. MCM7 is not phosphorylated by Cdc7 (see also the supplemental material). B, the wild-type (lanes 1, 5, and 9) and 6AA mutant version (lanes 2, 6, and 10) of GST-fused MCM4N protein (amino acid residues 1–198; 4 pmol) or control GST protein (lanes 3, 7, and 11; 4 pmol) was phosphorylated in vitro by the kinases indicated (Cdc7-ASK, 0.6 pmol; Cdc2-CyclinB, 0.1 pmol). Lanes 4, 8, and 12 represent reactions without a substrate. First and third panels, autoradiogram; second and fourth panels, silver-stained gel. The incorporation of ATP into the MCM4N substrates (wild-type or 6AA), estimated from radioactivity of the dried gel containing the protein band measured by a scintillation counter, was as follows (in pmol); lane 1, 0.31; lane 2, 0.27; lane 3, 0.23; lane 4, 0.06; lane 5, 0.89; and lane 6, 0.39. C, the in vitro kinase reaction was conducted as described for B except that radioactive ATP was not included. The reaction mixture was analyzed by Western blotting using the MCM4 S6T7 antibody. The reaction mixtures were analyzed on 7.5% SDS-PAGE (A) or 4–20% gradient SDS-PAGE (B and C).
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FIGURE 3. Effect of Cdc7 inactivation in mouse ES cells on phosphorylation of MCM4 protein observed in Triton-insoluble fractions. A, FACS analyses of DNA content of Cdc7+/+ (left) and Cdc7−/−tg ES cells (right) at 3.5 days (3.5d) after Ad-Cre infection. B, Triton-soluble (S) and -insoluble (P) extracts were prepared before infection (lanes 1–4), at 2.5 days (lanes 5–8) or 3.5 days (lanes 9–12) after infection. Proteins were analyzed by Western blotting using the antibody indicated. The open arrowheads indicate the mobility-shifted forms of MCM4 protein, which are generated by Cdc7-dependent phosphorylation, while the closed arrowheads indicate the non-shifted form. C, Triton-insoluble fractions prepared from Cdc7+/+ (lane 2) or Cdc7−/−tg (lanes 1 and 3) before infection (lane 1) or at 3.5 days after infection (lanes 2 and 3) were analyzed by Western blotting using the antibodies indicated. Two isoforms of Cdc7 are detected in the wild-type ES cells (B, lanes 1, 2, 5, 6, 9, and 10; C, lane 2; indicated by star marks), while only the smaller one is expressed in Cdc7−/−tg ES cells (B, lanes 3 and 4; C, lane 1; Ref. 36), which disappeared upon Ad-Cre infection (B, lanes 11 and 12; C, lane 3).

The highly mobility-shifted forms of MCM4, which can be detected in the Triton-insoluble fraction in the wild-type ES cells, disappeared upon the infection of Cre-encoding adenoviruses (Ad-Cre) on Cdc7−/−tg cells, which efficiently excises out the transgene (tg) and induces the loss of the Cdc7 genes (Fig. 3B). This strongly suggests that the characteristic phosphorylation of MCM4 observed during the S phase and on the chromatid is caused by Cdc7. We then examined the phosphorylation state of the serine 6 and threonine 7. The S6T7 band disappeared in the chromatin-enriched fraction from Ad-Cre-transfected Cdc7−/−tg cells (Fig. 3C, lane 3).

We also examined in human cells the effect of Cdc7 down-regulation on the S6T7 phosphorylation. HeLa cells were transfected with Cdc7-specific siRNA and were further treated with HU to enrich the S phase cell population. FACS analyses of DNA content of each cell population indicated that these treatments did not significantly affect the cell cycle profile of the cells analyzed (Fig. 4B). Treatment of HeLa cells with Cdc7 siRNA reduced the intensities of highly mobility-shifted forms of MCM4 in Triton-insoluble, chromatin-enriched fractions. Furthermore, in the same fraction, the level of S6T7 phosphorylation was significantly reduced (Fig. 4A; see also lane 3 of Fig. 4B). We also examined the effect of purvalanol A, a specific inhibitor for Cdk. The addition of purvalanol A significantly reduced the phosphorylated forms of MCM4 (data not shown). The S6T7-reactive band also diminished much after purvalanol A treatment (Fig. 4B, lanes 2 and 4). Combination of purvalanol A and Cdc7 siRNA led to almost complete loss of S6T7 phosphorylation (Fig. 4B, lane 4). These results strongly suggest that the phosphorylation of S6T7 of MCM4 depends on both Cdk and Cdc7 functions.

We next examined whether the overexpression of Cdc7-ASK may enhance the level of S6T7 phosphorylation in vivo. We transiently transfected 293T cells with plasmids expressing Cdc7 and ASK (9, 37). Upon transfection of Cdc7 and ASK, the amount of the mobility-shifted forms of MCM4 increased, and the S6T7 signal also was significantly enhanced (Fig. 5A, lane 2). We also stained the transfected cells with the S6T7 antibody and found that the immunofluorescence signals are greatly increased after transfection of Cdc7 and ASK plasmids (Fig. 5B). In contrast, transient transfection of Cdk2 enhanced T7 antibody signal but not the S6T7 signal (Fig. 5A, lane 3). These results are consistent with the hypothesis that Cdk mainly phosphorylates T7 and Cdc7 phosphorylates S6.
MCM4 Interacts with Cdc45 during S Phase—Cdc45 is a key protein required for initiation of DNA replication. The loading of Cdc45 onto chromatin depends on the actions of two kinases, namely cyclin-dependent kinase and Cdc7 kinase (38–41). In Xenopus egg extracts, inactivation of Cdk or depletion of Cdc7 blocks the chromatin binding of Cdc45 protein (6, 24). Cdc45 is detected in Triton-soluble fractions throughout the cell cycle in HeLa cells, but a small portion of this protein appears in the chromatin fractions during S phase (0–6 h and 18–24 h after release from double thymidine block, Fig. 1B). Since MCM4 is a chromatin component required for initiation of DNA replication, we have examined whether MCM4 interacts with Cdc45.

To examine the interaction of MCM4 with Cdc45, immunoprecipitation with MCM4 antibody was conducted from the chromatin-enriched extracts prepared by sonication of the Triton-insoluble pellets (Fig. 6A). HeLa cells enriched in S and G2 phases were prepared by release from double thymidine block for 3 and 10 h, respectively. In these fractions, the hyperphosphorylated forms of MCM4 as well as the S6T7-reacting protein increased in cells released for 3 h from double thymidine block, and the MCM4 immunoprecipitate contained Cdc45 protein, the amount of which increased in the S phase extract (Fig. 6A, lane 1). Thus, MCM4 protein interacts with Cdc45 in the chromatin fraction during S phase, consistent with the expected role of MCM in facilitating the loading of Cdc45 protein onto chromatin for initiation of DNA replication. This is consistent with the previous results reported in yeasts (40–42).

We prepared the chromatin-enriched fractions also from U2OS cells enriched in early S phase by mimosine or HU treatment. The immunoprecipitates with anti-Cdc45 antibody from these fractions contained MCM4 protein. Notably, mobility-shifted (thus phosphorylated) forms, which also reacted with the S6T7 antibody, were enriched in the immunoprecipitates in mimosine- or HU-treated cell extracts (Fig. 6B, lanes 2 and 3, top and middle panels). FLAG-tagged Cdc45 protein transiently expressed in U2OS cells also coimmunoprecipitated more preferentially with the mobility-shifted forms of MCM4 (data not shown). These results indicate that Cdc45 protein interacts more preferentially with the phosphorylated forms of MCM4 in the chromatin fractions.

We next examined the effect of Cdc7 depletion on chromatin association of Cdc45 and MCM4-Cdc45 interaction. When HeLa cells were treated with Cdc7 siRNA for 48 h, the amount of Cdc45 protein in the Triton-insoluble fraction was significantly reduced in both asynchronous culture or in mimosine-treated cells. This effect is not due to the cell cycle effect, since the cell cycle profiles are almost identical between Cdc7 siRNA-treated cells and control cells (lanes 1, 2, 4, 5, 7, and 8 in Fig. 6C). The amounts of Cdc45 protein in the double thymidine block-release cells, which are in S phase, were only slightly reduced.
This is due to the incomplete depletion of Cdc7 protein, since the siRNA treatment was only 24 h in these cells (see supplemental Fig. S2).

We then examined the interaction of MCM4 and Cdc45 in the Cdc7 siRNA-treated cells. We used the double thymidine block release S phase cell extracts. The amount of Cdc45 protein coimmunoprecipitated with MCM4 was clearly reduced in Cdc7 siRNA-treated cells (lanes 3 and 4 in Fig. 6D). These results indicate that Cdc7 is required for efficient chromatin association of Cdc45 protein during S phase, and it is likely that this association may be caused at least partly through interaction with phosphorylated forms of MCM4.

To further test this possibility, we then examined interaction of mutant forms of MCM4 with Cdc45. The wild-type, 6AA, or 6EE mutant form of the FLAG-tagged MCM4 protein was transiently expressed in U2OS cells, and Triton-soluble and -insoluble extracts were prepared. Immunoprecipitation with anti-Cdc45 antibody resulted in efficient coimmunoprecipitation of the 6EE form of MCM4, which mimics the phosphorylated form of MCM4 but not the unphosphorylatable 6AA mutant form of MCM4, both in Triton-soluble and -insoluble fractions. The wild-type form was coimmunoprecipitated to an intermediate level (Fig. 6E, lanes 1–3). These results further support our conclusion that
Cdc45 more preferentially interacts with the phosphorylated forms of MCM4 protein.

Cdc45 Interacts with MCM Proteins in a Cdc7-dependent Manner in Fission Yeast—Since the presence of the clusters of serine and threonine residues in the N-terminal segment of MCM4 is conserved in yeasts (Fig. 7A), we have examined the interaction of Cdc45 with MCM in fission yeast cells. We previously constructed a mutant fission yeast strain, hsk1–89, which is temperature-sensitive for Hsk1 kinase, the Cdc7 homologue in fission yeast. In this mutant, Hsk1 kinase activity is significantly reduced (37). The wild-type (hsk1+) and hsk1–89 cells were grown at 25 °C, a permissive temperature for the mutant, and treated with HU to enrich S phase cell population. Chromatin-enriched fractions were prepared and MCM4 and MCM6 proteins were examined by Western blotting. A mobility-shifted form of MCM4 (Cdc21), which is enriched in HU-treated cells disappeared by prior phosphatase treatment, and the amount of this form was greatly diminished in hsk1–89 cells (Fig. 7B, upper panel). MCM6 (Mis5) protein also appeared as a doublet, and the upper band disappeared by the phosphatase treatment and was again reduced in its quantity in hsk1–89 cells (Fig. 7B, lower panel). Similar mobility shift was not detected with Mis5/H9004 protein lacking the N-terminal 47 amino acids containing 12 serine and threonine residues (Fig. 7B, lanes 9 and 10). These results indicate that both MCM4 and MCM6 proteins are phosphorylated in a manner dependent on Hsk1 kinase activity and that the N-terminal 47 amino acids of MCM6 (Mis5) containing a cluster of serine and threonine residues are responsible for the mobility shift.

Since the strain used expressed HA-tagged Goa1 (fission yeast Cdc45; 42), Cdc45 was immunoprecipitated by anti-HA antibody and the immunoprecipitates, together with the

**FIGURE 7.** Conservation of serine/threonine residues in the N-terminal segments of MCM2, MCM4, and MCM6 proteins and interaction of MCM proteins and Cdc45 in fission yeast in a manner dependent on the Hsk1 functions. A, the sequences of N-terminal segment from MCM2, MCM4, and MCM6 proteins from human, Xenopus, budding yeast, and fission yeast are shown. The serine and threonine residues are indicated in red, and (S/T)(S/T)P sequences are underlined. The end points of some of the fission yeast MCM4 and MCM6 N-terminal truncation mutants are indicated. B, Goa1-HA3 hsk1+ (lanes 1–3 and 7–10) and Goa1-HA3 hsk1–89 (lanes 4–6) yeast cells were grown in yeast extract-supplemented medium at 25 °C and were treated with 11 mM HU for 4 h before harvest. The cells were treated with Zymolyase and the resulting spheroplasts were lysed by Triton. The Triton-insoluble pellet (chromatin-enriched) fractions were solubilized by sonication, and the recovered proteins were analyzed by Western blotting. Lanes 9 and 10, Δ47Mis5 background. The extracts were pretreated with phosphatase at 30 °C for 30 min (lanes 3, 6, 8, and 10) or at 30 °C in the presence of phosphatase inhibitors (50 mM NaF and 0.1 mM Na3VO4; lanes 2 and 5). They were analyzed by Western blotting using anti-Cdc21 (upper) and anti-Mis5 (lower) antibodies. C, the extracts were prepared as described for B, and Cdc45 (Goa1) protein was immunoprecipitated by HA antibody. The input extract (I, 40%) and the immunoprecipitates (P) were analyzed by the Western blotting to detect the proteins indicated. In B and C, black and gray arrowheads indicate the positions of non-shifted and shifted (phosphorylated) forms, respectively, of the proteins indicated, and star marks indicate the degradation products of MCM4 (Cdc21) and MCM6 (Mis5) proteins. WT, wild-type.
input extracts, were analyzed by Western blotting (Fig. 7C). In the wild-type cells, coimmunoprecipitation of MCM4 (Cdc21), MCM5 (Nda4), and MCM6 (Mis5) was observed. Notably, the slow migrating forms, presumably generated by phosphorylation, were selectively coimmunoprecipitated in the case of MCM4 and MCM6. In contrast, the immunoprecipitates contained very little MCM proteins in hsk1–89 cells, although Cdc45 protein was present. This result indicates that Cdc45 and MCM interact on the chromatin in a manner dependent of the Cdc7 function in the fission yeast cells as well and that the phosphorylated forms of MCM4 and MCM6 specifically interact with Cdc45, as was found in human cells.

The Sufficient Level of Phosphorylation in the N-terminal Segment of MCM4 May Be Required for the Optimum Growth of Fission Yeast Cells—To assess the biological significance of the phosphorylation of MCM4 by Cdc7 kinase in vivo, we have generated mutant MCM4 proteins in which all the seven (S/T)/((S/T)/T)P sequences in the N-terminal 129 amino acids of fission yeast MCM4 protein were replaced with AAP or EEP sequence. Both AAP and EEP mutants proteins ectopically expressed under the nmt1 promoter on a plasmid were able to rescue the temperature-sensitive growth of cdc21-M68 strain (in the presence of thiamine, which represses the nmt1 promoter), indicating that the mutation does not affect the essential function of MCM4 protein (Table 1). We then generated N-terminal truncation mutants lacking either 67, 130, 150, or 200 amino acids of MCM4 protein (Fig. 7A) and examined their functions. The N-terminal truncated mutant lacking a 67-, 130-, or 150-amino acid segment, containing clusters of the serine/threonine residues presumably phosphorylated by Cdc7 and Cdk, was able to rescue the growth of cdc21-M68 at 37 °C in the absence of thiamine (Fig. 8A and Table 1), although further deletion up to 200 amino acids rendered the mutant deficient in complementation. Since the residues conserved in MCM4 start at position 158, the defect of the Δ200 mutant may be due to loss of some essential conserved residues for MCM4 function. Whereas Δ67cdc21 and Δ130cdc21 complemented the growth even in the presence of thiamine, Δ150cdc21 did not show efficient complementation under this condition (Fig. 8A). We then integrated these N-terminally truncated cdc21 to replace the cdc21− wild type allele. Δ130 was able to grow with growth rate similar to the wild-type cells in the absence of thiamine and showed no sensitivity to UV or HU treatment. However, it did not grow in the presence of thiamine (Fig. 8B and data not shown). Further deletion up to 150 amino acids was not recovered under the same condition (even in the absence of thiamine; data not shown). The above results indicate that the deletion of N-terminal 130 amino acids of MCM4 may be tolerated, but further 20 amino acids are required for the optimum growth.

There are seven serine or threonine residues between the positions 131 and 150 of fission yeast MCM4. Therefore, reduced viability of Δ150 may be due to reduced level of phosphorylation. We then examined whether phosphorylation sites from other molecules can restore the viability of the Δ150 mutant. We have inserted the N-terminal 42 amino acids (1–42) of MCM6, which we showed is a target of Hsk1-mediated phosphorylation (Fig. 7B). The resulting MCM6Δ150 on a plasmid could restore the growth of cdc21-M68 at 37 °C even in the presence of thiamine (Fig. 8C). There is only one TP site and no other consensus Cdk phosphorylation sites within the inserted MCM6 sequence. Thus, the results strongly suggest that a sufficient level of phosphorylation at the N terminus of MCM4, most likely by Cdc7, is required for the optimum growth.

The Phosphorylation of the N-terminal Serine/Threonine-rich Segments of MCM May Play Redundant Roles in Initiation of DNA Replication—Serine and threonine residues are enriched in the N-terminal region of MCM2 protein as well (12 out of 50, 10 out of 44, 8 out of 60, and 11 out of 58 in, respectively, human, Xenopus, budding yeast, and fission yeast MCM2 N-terminal segment; Fig. 7A). The results in fission yeast as well as in mammalian cells strongly indicate that the N-terminal segment of MCM2 is phosphorylated by Cdc7 kinase (17, 20). Therefore, we have generated a mutant MCM2 in which 10 serine and threonine residues present within its N-terminal 35 amino acid segment were replaced with alanine (Cdc19−10A) or glutamic acid (Cdc19−10E; Fig. 7A). Both mutants, expressed on a plasmid in the presence of thiamine, were able to rescue the growth of cdc19-P1 cells at 37 °C, and sensitivity to UV and HU was not affected by the mutations (Table 1 and data not shown). These results indicate that the phosphorylation of the N-terminal segment of MCM2 is not essential for cells’

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**TABLE 1**

| MCM2               | Complementation of cdc19-P1 |
|--------------------|-----------------------------|
| Cdc19−10A (plasmid)| + +                         |
| Cdc19−10E (plasmid)| + +                         |

| MCM4               | Complementation of cdc21-M68 |
|--------------------|-----------------------------|
| AAP7cdc21 (plasmid)| + +                         |
| Cdc19−10A (plasmid)| + +                         |
| Δ67cdc21 (plasmid) | + +                         |
| Δ130cdc21 (plasmid)| + +                         |
| Δ150cdc21 (plasmid)| + +                         |
| Δ200cdc21 (plasmid)| + +                         |
| Δ67cdc21 (genome)  | Cell growth Viable         |
| Δ130cdc21 (genome) | + +                         |

| MCM6               | Complementation of mcm2 mcm4 mcm6 (cdc19-P1 background) |
|--------------------|----------------------------------------------------------|
| Cdc19−10A (plasmid)| + +                         |
| Cdc19−10E (plasmid)| + +                         |
| Δ47mis5 (genome)   | Cell growth Viable         |

| MCM2 mcm4 mcm6 (cdc19-P1 background) | Complementation of mcm2 mcm4 mcm6 (cdc19-P1 background) |
|-------------------------------------|----------------------------------------------------------|
| Cdc19−10A (plasmid)                | + +                         |
| Cdc19−10E (plasmid)                | + +                         |
| Δ47mis5 (genome)                   | Cell growth Viable         |
| Δ47mis5 (genome)                   | Unviable?                  |

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**Cdc7-mediated Phosphorylation of MCM4**
We have constructed a cell expressing only a mutant MCM6 lacking the N-terminal 47 amino acid segment containing nine serine and three threonine residues. This mutant cell (Δ47mis5), which does not undergo the phosphorylation-induced mobility-shifted, was viable and did not show any sensitivity to UV and HU (Table 1 and data not shown).

Finally, we have crossed the Cdc19–10A Δ130cdc21 and Δ47mis5 (under cdc19-P1 background) to examine whether the triple mutant is viable. However, we were not able to recover the strain carrying the three mutations even at 25 °C, suggesting that the combination of the three mutations may result in loss of viability (Table 1). Although we cannot rule out the possibility that combinations of these alanine substitution and N-terminal truncation mutations of MCM (in conjunction with cdc19-P1 mutation) may somehow disrupt the structures and functions of the MCM complexes, these results are consistent with the conclusion that the phosphorylation of the N-terminal non-conserved tails of MCM2, MCM4 or MCM6 plays redundant roles in initiation of DNA replication, presumably in recruitment of the replication factors including Cdc45.

**DISCUSSION**

Cdc7 kinase is evolutionally conserved and is known to play crucial roles in initiation and progression of DNA replication. It phosphorylates MCM subunits in vitro. MCM2 has been shown to be phosphorylated in a manner dependent on Cdc7 function in yeasts (15, 23). MCM4 was previously reported to be phosphorylated in Xenopus egg extracts (32). It was also reported that the fission yeast MCM4 in a MCM complex is phosphorylated by Hsk1 kinase in vitro (25).

In this report, we have shown that specific residues of the MCM4 N-terminal segment can be phosphorylated by Cdc7 kinase both in vivo and in vitro. Serine and threonine residues are highly enriched in the N-terminal segments of MCM4 proteins (36 out of 151, 27 out of 146, 48 out of 179 and 51 out of 158 in, respectively, human, Xenopus, budding yeast, and fission yeast MCM4; Fig. 7A). We also noticed the presence of multiple copies of (S/T)(S/T)P in the N-terminal region of MCM4 proteins (6, 3, 5 and 7 copies in the above segments of human, Xenopus, budding yeast, and fission yeast MCM4, respectively; Fig. 7A). We have identified one of these sites as a target of Cdc7 kinase. We speculate that Cdc7 phosphorylates multiple (S/T)(S/T)P as well as other serine/threonine residues within the N-terminal segment of MCM4, causing a significant mobility shift on SDS-PAGE. In fact, the substitution of the six sets of (S/T)(S/T)P with EEP led to similar mobility shift on its own, strongly suggesting that phosphorylation of multiple sites contributes to the generation of a series of mobility-shifted forms on SDS-PAGE. A number of Cdk target sites have been identified in the same N-terminal segment of MCM4 and phosphorylation of these residues have indeed been shown using phosphorylation-specific antibodies (44). Many of these sites overlap with the second serine or threonine of (S/T)(S/T)P, strongly suggesting that Cdk is responsible for this phosphorylation. In fact, the T7 antibody, recognizing the phosphorylated threonine of STP (positions 6–8), mainly detects the highly mobility-shifted forms of MCM4 in the Triton-soluble frac-
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MCM4 (Δ150) resulted in growth inhibition. Since Cdk sites on MCM4 can be mutated without affecting viability (47), it is likely that loss of Cdc7-mediated phosphorylation is responsible for the phenotype associated with N-terminal truncation of MCM4. Consistent with this speculation, addition of 42 amino acids derived from MCM6, likely to be phosphorylated by Cdc7 but not containing a typical Cdk consensus site, to the Δ150 mutant of MCM4 restored the efficient growth of the Δ150 mutant. The absence of any conserved sequences containing serine and threonine in the N-terminal segments of MCM2, MCM4, and MCM6 of various species but the conserved presence of serine/threonine clusters suggest that the recruitment of Cdc45 may depend on the negatively charged segments of the MCM N-terminal tails, generated by Cdc7-mediated phosphorylation, and not on particular sequence motifs.

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