Inhibition of Mcm4,6,7 Helicase Activity by Phosphorylation with Cyclin A/Cdk2*

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A strong body of evidence indicates that cyclin-dependent protein kinases are required not only for the initiation of DNA replication but also for preventing over-replication in eukaryotic cells. Mcm proteins are one of the components of the replication licensing system that permits only a single round of DNA replication per cell cycle. It has been reported that Mcm proteins are phosphorylated by the cyclin-dependent kinases in vivo, suggesting that these two factors are cooperatively involved in the regulation of DNA replication. Our group has reported that a 600-kDa Mcm4,6,7 complex has a DNA helicase activity that is probably necessary for the initiation of DNA replication. Here, we examined the in vitro phosphorylation of the Mcm complexes with cyclin A/Cdk2 to understand the interplay between Mcm proteins and cyclin-dependent kinases. The cyclin A/Cdk2 mainly phosphorylated the amino-terminal region of Mcm4 in the Mcm4,6,7 complex. The phosphorylation was associated with the inactivation of its DNA helicase activity. These results raise the possibility that the inactivation of Mcm4,6,7 helicase activity by Cdk2 is a part of the system for regulating DNA replication.

In eukaryotic cells, a number of proteins are involved in the initiation of DNA replication including ORC, Cdc6, Mcm, Cdc45 proteins, cyclin-dependent protein kinase, and Cdc7 kinase (1). Both ORC and Cdc6 are required for the loading of Mcm proteins onto chromatin and the replication origin (2–6). Cyclin-dependent protein kinases are necessary for the initiation of eukaryotic DNA replication (7–9), which has been shown in vivo in yeast (10–13) as well as in vitro in Xenopus egg extracts (14, 15) and human cell extracts (16). It has been reported that cyclin-dependent kinase is involved in loading Cdc45 onto chromatin in Saccharomyces cerevisiae (17) and in Xenopus egg extracts (18), and Cdk regulates the activity of human DNA polymerase α/primase in vitro (19). The cyclin-dependent kinase is also required for preventing reinitiation of DNA replication in a single cell cycle in yeast cells (20–25). Mcm proteins, six in all, are conserved from yeast to mammals and play an essential role in DNA replication (26). They are also a component of the replication licensing system proposed by Blow and Laskey (27), which permits only a single round of DNA replication per cell cycle in Xenopus egg extracts (28–30).

Mcm proteins bind to chromatin before the initiation of DNA replication and detach from it as the replication proceeds (31–33). These findings suggest that the binding of Mcm proteins confers to the chromatin the ability to replicate once in a cell cycle, and Mcm proteins that have completed the function in DNA replication are inactivated during S phase (34). Mcm proteins are phosphorylated during S and G2/M phases (32, 35, 36). Cyclin B/Cdc2 complex is mainly responsible for the phosphorylation of Mcm4 at G2/M phase (37–39). Consistent with this notion, several consensus sites of cyclin-dependent kinases are clustered in the amino-terminal region of Mcm4. Thus, it appears that Mcm proteins and cyclin-dependent protein kinases are cooperatively involved in restricting DNA replication once in a cell cycle. However, the molecular mechanism underlying this regulation remains to be analyzed.

All of the Mcm proteins have a common DNA-dependent ATPase motifs in the central domain (40). Consistent with this, our group recently showed that Mcm4,6,7 proteins form a 600-kDa complex and function as a DNA helicase in vitro (41). In addition, we showed that Mcm2 inhibits the DNA helicase activity by converting the 600-kDa Mcm4,6,7 complex to a 450-kDa Mcm2,4,6,7 complex (42). To understand the interplay between Mcm proteins and cyclin-dependent kinase during S phase, we examined the effect of phosphorylation with cyclin A/Cdk2 on the DNA helicase activity of the Mcm4,6,7 complex. The results show that the kinase phosphorylates Mcm4 in the Mcm4,6,7 complex and thereby inactivates its DNA helicase activity. These findings suggest that Mcm4 in the Mcm complex is a crucial substrate of the kinase that plays an essential role in preventing over-replication.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Human Mcm4,6,7 and Mcm2,4,6,7 complexes were purified from HeLa cells by histone-Sepharose column chromatography and glycerol gradient centrifugation as reported (41). Cyclin A/Cdk2 complex was purified from HgH5 cells co-infected with the recombinant baculoviruses (16) (kindly provided by R. A. Laskey) by nickel-nitrilotriacetic acid column (Qiagen) and then mono Q column chromatography. The concentration of the kinase was approximately 100 units/μl, and the protein concentration was 170 μg/ml. One unit of kinase activity was defined as the activity that phosphorylates 1 pmol of histone H1 for 30 min at 30 °C under the condition where one microgram of H1 histone was incubated in 40 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, and 10 μM ATP. Mouse p27Kip1 was over-produced in Escherichia coli (BL21) in which p27Kip1 gene had been cloned into pGEX6P (Amersham Pharmacia Biotech). The GST-p27Kip1 fusion protein was purified from extracts of E. coli by using glutathione-Sepharose beads, and p27Kip1 was obtained by digestion of the fusion protein with PreScission Protease as the manufacturer suggested.

Production of Fusion Proteins—Various regions of mouse Mcm4 protein were produced in E. coli as thiouc fusion proteins (Invitrogen). Mcm4 genes were cloned into a pTrxFus vector at KpnIXbaI sites. Soluble fusion protein (4/4, a quarter fragment of Mcm4 from the
carboxyl-terminal end) was prepared as the manufacturer suggested. Insoluble forms of the fusion protein were recovered by lysing collected cells with 6 mM guanidium chloride, and the supernatant of the lysed cells (0.5 μl) was used for an in vitro phosphorylation reaction. The production of these fusion proteins was confirmed by Western blotting using an anti-thiofus antibody.

In Vitro Phosphorylation and DNA Helicase Assay—The Mcm complexes and the Mcm4 fusion proteins were incubated with various amounts of the cyclin A/Cdk2 complex at 37 °C for 30 min in a 20-μl reaction mixture consisting of 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 10 mM MgCl₂, 1 mM ATP, and 0.01% Triton X-100 in the presence of [γ-32P]ATP. The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. For the coupled reactions of phosphorylation and DNA helicase, a 10-μl solution, which contained 40 mM Tris-HCl, pH 7.9, 20 mM 2-mercaptoethanol, 10 mM ATP, 0.5 mg/ml bovine serum albumin, and an end-labeled 17-mer oligonucleotide annealed to M13 mp18 DNA (5 fmol), was added to the phosphorylation reaction, and the incubation was continued for 45 min. The DNA was analyzed in 12% polyacrylamide gel containing TBE.

The phosphorylation and gel shift analysis in Fig. 2E, the following reactions were constructed. After phosphorylation of Mcm4,6,7 complex with Cdk2 for 30 min at 37 °C in a mixture (20 μl) containing 20 mM Hepes, pH 7.5, 30 mM NaCl, 10 mM MgCl₂, 0.1 mM ATP, and 0.01% Triton X-100, a mixture (15 μl) containing 10 mM creatine phosphate (sodium salt, pH 7.7, 10 mM ATP, 15 mM potassium phosphate, 0.01% Triton X-100 and 0.15 μmol of an endo-labeled 37-mer oligonucleotide (5′-AATTCGAGCTCGTACCCGGGGATCCTCTAGAGTCGGA-3′) were added, and the incubation was continued for 30 min. 10% glutaraldehyde was added to the reaction mixture at a final concentration of 0.1%, and the reaction was incubated for additional 10 min. The reaction mixture was analyzed with a 5% polyacrylamide gel under non-denaturing conditions. The gel was dried on DES2 paper (Whatman), and the radioactivity was analyzed by using a Bio-Image analyzer (Fuji).

In Vivo Labeling—Logarithmically growing HeLa cells were incubated with 1 μM hydroxyurea for 18 h to synchronize the cells at G1/S to S phase. After removal of hydroxyurea, cells were cultured in phosphate-free minimum essential medium containing 10% dialyzed calf serum for 1 h. 1 μCi of orthophosphate was added to the medium, and the incubation was continued for 2 h. Proteins were extracted from the cells by suspending with CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1 mM β-mercaptoethanol, 0.01% Triton X-100, 0.1% glycine (pH 2.5) containing 0.1 M NaCl). After being neutralized, proteins were analyzed in 10% polyacrylamide gel containing SDS.

Digestion with Lysyl Endopeptidase—Mcm proteins labeled in vitro or in vivo were digested with lysyl endopeptidase (2 μg/ml) (Wako, Japan) for 1 or 16 h at 37 °C in the presence of 0.1% SDS, and the peptides were analyzed in 20% polyacrylamide gel containing SDS.

Construction of Mouse Mcm4,6,7 Complex Containing Mutated Mcm4—To produce deletion mutants of Mcm4 protein, polymerase chain reaction primer to add a histidine tag at the amino-terminus was used for the construction of deletion mutants of Mcm4 protein. To produce deletion mutants of Mcm4 protein, polymerase chain reaction primer to add a histidine tag at the amino-terminus was used. The Mcm4,6,7 complex was purified by nickel column chromatography.

RESULTS

Phosphorylation of Mcm Proteins with Cyclin A/Cdk2—Several lines of evidence suggest that Mcm proteins are substrates of cyclin-dependent kinases in vitro (37–39). Mcm2 and 4 proteins were phosphorylated during S phase of cell cycle when cyclin A/Cdk2 among cyclin-dependent kinases is the most active. To know the functional relationship between Mcm and these kinases, the Mcm4,6,7 complex purified from HeLa cells (Fig. 1A, left), which has a DNA helicase activity, was incubated with a human cyclin A/Cdk2 complex under the conditions described under “Experimental Procedures.” The purified cyclin A/Cdk2 complex mainly consisted of two proteins of approximately 40 and 50 kDa on SDS-polyacrylamide gel (Fig. 1D), those that were recognized with anti-human Cdk2 and anti-human cyclin A antibodies, respectively (data not shown). Approximately the 100-kDa protein, which is probably Mcm4 or 6 protein, was preferentially phosphorylated with the kinase (Fig. 1B, left). The phosphorylated proteins were extracted from the position of 100–110 kDa in the presence of a high concentration of the kinase. These phosphorylated proteins were blotted to the filter and proved with anti-Mcm4 antibodies (Fig. 1C). The Mcm4 protein detected was also expanded to the higher molecular weight position in the presence of a high concentration of the kinase; the change in the mobility of Mcm4 higher molecular weight position in the presence of a high concentration of the kinase (data not shown). These results indicate that Mcm4 protein in the Mcm4,6,7 complex is...
preferentially phosphorylated by Cdk2 in vitro. This conclusion is consistent with the notion that Mcm4 protein has several consensus sites for cyclin-dependent kinase (see below). From the incorporated $^{32}$P, it was calculated that approximately two molecules of phosphate on average were incorporated into Mcm4 protein at maximum. Cdk2 also phosphorylated Mcm2 in addition to Mcm4 in the 450-kDa Mcm2,4,6,7 complex that is inactive for the function of DNA helicase (Fig. 1, A, right, and B, right). A similar level of phosphorylation was observed between Mcm2 and Mcm4 proteins. The mobility of the phosphorylated Mcm2 increased as the number of incorporated phosphate molecules increased. A similar change in the mobility with phosphorylation was observed in vivo for Mcm2 (32) and Mcm4 (36–39).

Inhibition of Mcm Helicase Activity by Phosphorylation—

Our group reported that a 600-kDa human Mcm4,6,7 protein functions as a DNA helicase. Moreover, we recently showed that the DNA helicase activity is intrinsically associated with the recombinant mouse Mcm4,6,7 complex, which was prepared from baculovirus-infected insect cells (43). We examined the effect of phosphorylation by cyclin A/Cdk2 complex on the DNA helicase activity of the human Mcm4,6,7 complex (Fig. 2A). After phosphorylation of Mcm4,6,7 complex (50 ng) with increasing amounts (0, 85, 175, and 350 units) of Cdk2, the mixture was incubated with the DNA for measuring the DNA helicase activity. The results showed that the DNA helicase activity of the Mcm4,6,7 complex was decreased to 10% of the original activity (Fig. 2B). In contrast, the DNA helicase activity of SV40 T antigen was not inhibited by the phosphorylation with Cdk2.
The reaction mixtures were incubated for 1 h under standard conditions in the presence or absence of cyclin A/Cdk2 and p27Kip1 as indicated. The DNA helicase activity of the complex was inhibited by the addition of Kip1, which is an inhibitor of the kinase. Under prior phosphorylation reaction (Fig. 3A). The inhibition of the DNA helicase activity of the Mcm4,6,7 complex was inhibited by the addition of cyclin A/Cdk2 (170 units) was performed in the absence or presence of Kip1, and phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. In the absence of Kip1, 1.3 mol of phosphate were incorporated into a 100-kDa band of Mcm4. In addition to the 100-kDa Mcm4 band, the kinase complex phosphorylated a 50–55-kDa band that is probably cyclin A.

![Figure 3](image)

**Fig. 3. Reverse of inhibition of DNA helicase activity by Kip1.** A, Mcm4,6,7 proteins (50 ng) were added to the DNA helicase reaction in the presence or absence of cyclin A/Cdk2 and p27Kip1 as indicated. The reaction mixtures were incubated for 1 h under standard conditions containing 10 mM ATP and 10 mM MgCl2. B, the phosphorylation of Mcm4,6,7 complex (50 ng) with cyclin A/Cdk2 (170 units) was performed in the absence or presence of Kip1, and phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. In the absence of Kip1, 1.3 mol of phosphate were incorporated into a 100-kDa band of Mcm4.

Under similar conditions (data not shown). The Mcm 4,6,7 proteins form a 600-kDa complex under nondenaturing conditions as well as under denaturing conditions after protein cross-linking (41, 42). A correlation of the DNA helicase activity with the amount of the 600-kDa Mcm4,6,7 complex but not a 450-kDa Mcm2,4,6,7 complex was observed (42). These findings indicate that the 600-kDa complex is a functional unit of the DNA helicase. After incubation of the Mcm4,6,7 complex with cyclin A/Cdk2, the Mcm complex was analyzed under nondenaturing conditions (Fig. 2C). In proportion to the amount of Cdk2 added, the mobility of the 600-kDa Mcm4,6,7 complex was slightly retarded, and in addition the amount of a smaller complex of about 400 kDa enhanced. The 600-kDa Mcm4,6,7 complex was indeed phosphorylated with cyclin A/Cdk2 (Fig. 2D). The effect of Cdk2 phosphorylation on single-stranded DNA binding activity of the Mcm4,6,7 complex was examined by gel shift analysis using a labeled 37-mer oligonucleotide (Fig. 2E). The result showed that the single-stranded DNA binding activity of Mcm4,6,7 complex was partially inhibited as the amount of Cdk2 increased. Thus, these results indicate that phosphorylation of the Mcm4,6,7 complex by Cdk2 kinase is associated with the structural change of the complex and also with the partial inhibition of its single-stranded DNA binding activity.

Relationship between phosphorylation of Mcm4,6,7 complex and inhibition of its DNA helicase activity was also examined by the following experiments. The DNA helicase activity of the Mcm4,6,7 complex was inhibited by the addition of cyclin A/Cdk2 from the start of the DNA helicase reaction without the prior phosphorylation reaction (Fig. 3A). The inhibition of the Mcm4,6,7 complex by cyclin A/Cdk2 was largely relieved by the addition of Kip1, which is an inhibitor of the kinase. Under similar conditions, Kip1 inhibited phosphorylation of Mcm4 in a dose-dependent manner (Fig. 3B). Thus, the inhibition of the kinase prevented the phosphorylation of Mcm4, and DNA helicase activity was not lost in these assays. These data support the conclusion that Cdk2 phosphorylates Mcm4 in the Mcm4,6,7 complex to inactivate its DNA helicase activity. Inhibition of the DNA helicase activity of Mcm4,6,7 complex by the phosphorylation with cyclinE/Cdk2 was also observed (data not shown).

**Sites of Mcm4 Phosphorylation**—The Mcm4 in the recombinant mouse Mcm4,6,7 complex was also phosphorylated, and the DNA helicase activity of the complex was inhibited by the phosphorylation, similar to the human Mcm4,6,7 complex. To determine the region of Mcm4 phosphorylated by cyclin A/Cdk2, various regions of mouse Mcm4 were produced as fusion proteins (Fig. 4A). These fusion proteins were incubated with the kinase (Fig. 4B). The fusion protein containing full size mouse Mcm4 (full) and the amino-terminal fragment of Mcm4 (1/4) were phosphorylated with cyclin A/Cdk2 (left) and then digested with lysyl endopeptidase in the presence of 0.1% SDS (right). Proteins were analyzed in 20% polyacrylamide gel containing SDS. Arrowheads indicate two phosphorylated peptides. D, HeLa cells were labeled with [32P]orthophosphate, and the extracts were immunoprecipitated with anti-Mcm4 or control antibody. Proteins eluted from the antibody beads were electrophoresed on 10% polyacrylamide gel containing SDS (left). The eluted proteins were digested with lysyl endopeptidase, and peptides were analyzed in 20% polyacrylamide gel containing SDS (right). In the left-hand lanes, the in vitro phosphorylated Mcm4,6,7 before and after digestion with the peptide were electrophoresed. Arrowheads indicate two peptides phosphorylated in vitro.

![Figure 4](image)

**Fig. 4. Cdk2 phosphorylates the amino-terminal fragment of Mcm4.** A, various regions of Mcm4, including four quarter fragments (1/4, 2/4, 3/4, and 4/4) and one fragment deleted of the amino-terminal quarter (2–4/4), were produced as fusion proteins with thiouc and analyzed by SDS gel electrophoresis together with purified human Mcm2,4,6,7 complex (left-hand lane). The fusion proteins were detected by using anti-thiofus antibody. B, the same proteins as in A were phosphorylated with cyclin A/Cdk2 in the presence of [γ-32P]ATP. Arrowheads in A and B indicate the bands of the amino-terminal quarter fragment of Mcm4. C, human Mcm2,4,6,7 complex (Mcmc) as well as fusion proteins containing full size mouse Mcm4 (full) and the amino-terminal fragment of Mcm4 (1/4) were phosphorylated with cyclin A/Cdk2 (left) and then digested with lysyl endopeptidase in the presence of 0.1% SDS (right). Proteins were analyzed in 20% polyacrylamide gel containing SDS. Arrowheads indicate two phosphorylated peptides. D, HeLa cells were labeled with [32P]orthophosphate, and the extracts were immunoprecipitated with anti-Mcm4 or control antibody. Proteins eluted from the antibody beads were electrophoresed on 10% polyacrylamide gel containing SDS (left). The eluted proteins were digested with lysyl endopeptidase, and peptides were analyzed in 20% polyacrylamide gel containing SDS (right). In the left-hand lanes, the in vitro phosphorylated Mcm4,6,7 before and after digestion with the peptide were electrophoresed. Arrowheads indicate two peptides phosphorylated in vitro.

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proteins suggests that these fragments are derived from an amino-terminal fragment of Mcm4 (amino acids 1–122) (Fig. 5B) that is connected with two amino acids from thiofus protein at the amino terminus (124 amino acids in total). The slowly migrating band increased in the presence of a high concentration of cyclin A/Cdk2, suggesting the band is more phosphorylated than the fast migrating band (data not shown). Almost all the radioactivity in the fusion protein containing full size Mcm4 was recovered in these two peptides after the digestion in Figure 4C. Mobility in the gel of the fast migrating band from human Mcm complex was slightly retarded comparing with the mobility of the band from mouse Mcm4 fusion proteins. This observation seems to be inconsistent with the notion that a peptide of 123 amino acids that is smaller than the peptide from mouse fusion proteins should be generated from human Mcm4. A band migrating at the position of approximately 35 kDa was recovered from the phosphorylated human Mcm2,4,6,7 complex, and this peptide is likely derived from the amino-terminal fragment of Mcm2 (amino acids 1–150). These results indicate that the amino-terminal region of Mcm4 in the Mcm4,6,7 complex is specifically phosphorylated by Cdk2. These findings are consistent with the fact that three consensus sites ((T/S)PXR/K) and eight possible sites ((T/S)PX) for cyclin-dependent kinases are clustered in this region of the mouse Mcm4 protein (Fig. 5B). These features are basically conserved from yeast to mammalian cells.

To examine whether this region is also phosphorylated in vivo, HeLa cells synchronized at G1/S and S phases with treatment of hydroxyurea were labeled with [32P]orthophosphate, and Mcm complexes were prepared from the cell extracts by using anti-Mcm4 antibodies. We first planned to prepare Mcm proteins bound to chromatin because Fujita et al. (44) and also Coue et al. (38) have reported that chromatin-bound form of Mcm4 protein is more phosphorylated than chromatin-unbound form of Mcm4 protein. In our hands, however, a large portion of total Mcm proteins was recovered in chromatin-unbound fraction, suggesting that not only the unbound Mcm proteins but also a significant portion of chromatin-bound Mcm proteins were collected in the same fraction. This fraction was used for immunoprecipitation experiments. A 100-kDa band of Mcm4 was recovered as a phosphorylated band, in addition to a 120-kDa protein that is most likely Mcm2 in the immunoprecipitates of anti-Mcm4 antibodies but not of control antibodies (Fig. 4D, left). When the immunoprecipitates of anti-Mcm4 antibodies were digested with lysyl endopeptidase, one major band whose mobility is almost the same as the upper band from
the digests of the Mcm proteins phosphorylated in vitro and a minor band corresponding to the lower band from the digests of the in vitro reaction were detected (Fig. 4D, right). Approximately 40% of the phosphate in the Mcm4 protein labeled in vivo were recovered in these smaller bands after the digestion with the peptidase. Phosphorylated peptides were also detected at the positions of 15 kDa as well as 50 kDa. These results suggest that the amino-terminal region of Mcm4 is phosphorylated in vivo as well as in vitro, and more than one molecule of phosphate is incorporated into this region of Mcm4.

To determine the in vitro phosphorylation sites of Mcm4 more precisely, we performed the following experiments. Recently, we succeeded in preparing a mouse Mcm6,7 complex, which is active for DNA helicase activity, by using a baculovirus expression system (43). We constructed two deletion mutants of Mcm4 (Δ(1–35) and Δ(1–112)) where the amino-terminal region of Mcm4 was removed. Two consensus sites of cyclin-dependent kinase were removed from the Mcm4 mutant of Δ(1–35) and all three sites were deleted from the mutant of Δ(1–112) (Fig. 5A). The amino acid sequence in this region is shown in Fig. 5B. These Mcm4 proteins were co-expressed with wild type Mcm6 and 7 proteins in insect cells. Approximately 500–550-kDa complexes containing Mcm4, 6, and 7 proteins were obtained (Fig. 5C and data not shown). These mutant complexes were phosphorylated with cyclin A/Cdk2 (Fig. 5D). A greatly reduced level (90% reduction) of phosphorylation was observed in the mutant complex containing Mcm4 of Δ(1–112), but a similar level of phosphorylation was detected in the mutant complex containing Mcm4 of Δ(1–35), suggesting that a region from amino acids 36 to 112 of Mcm4 is important for the phosphorylation with Cdk2. Cdk phosphorylated a 100-kDa band in the mutant complex containing Mcm4 of Δ(1–112); the band is probably a phosphorylated Mcm6. Comparison of phosphorylation between the wild type complex and the mutant complex containing Mcm4 of Δ(1–112) suggests that the level of the Mcm6 phosphorylation is approximately one-sixth of that of Mcm4 phosphorylation. Next, we site-directed mutagenized all of the three consensus sites (amino acids 7, 32, and 109) of cyclin-dependent kinase in full size Mcm4 (Ser and Thr were converted to Ala) and prepared the Mcm4,6,7 complex containing the mutated Mcm4. The level of Mcm4 phosphorylation in this mutant complex was comparable with that of the complex containing wild type Mcm4 (Fig. 5E). These results indicate that a region near the amino terminus is required for phosphorylation of Mcm4 with cyclin A/Cdk2, but the consensus sites are either unimportant or dispensable for the phosphorylation.

**DISCUSSION**

This study shows that cyclin A/Cdk2 efficiently phosphorylates Mcm4 in the Mcm4,6,7 complex, which is associated with the inhibition of the DNA helicase activity of the complex. The DNA helicase activity of the Mcm4,6,7 complex is probably necessary for the initiation of DNA replication. It has been suggested that cyclin-dependent kinase and Mcm proteins are cooperatively involved in the regulation of DNA replication. Thus, the findings of the present study raise the possibility that the inactivation of Mcm helicase activity by cyclin A/Cdk2 is a constituent of this regulatory system.

Mcm4 is phosphorylated during the cell cycle, as was shown in Xenopus egg extracts and mouse cells. At G2/M phase in Xenopus, Mcm4 is highly phosphorylated by a cyclin B/Cdk2 complex, which has been shown by Coute et al. (38). They reported that a chromatin-unbound form of Mcm4 is hypophosphorylated at early S phase, and in contrast, almost all of the chromatin-bound form of Mcm4 is partially phosphorylated: the level of the latter appears to be similar to that detected in the present study. Similar results have been obtained in mouse FM3A cells by Fujita et al. (39). They have observed that chromatin-bound forms of Mcm4 are more phosphorylated than the chromatin-unbound form of Mcm4 at S phase in FM3A cells. In addition, they have shown that the phosphorylation of mouse Mcm4 at G2/M phase is due to the cyclin B/Cdk2 complex using a temperature-sensitive mutant of the kinase. Although the kinase responsible for the phosphorylation of Mcm4 at S phase remains to be determined, it is likely that cyclin A/Cdk2 is involved. Thus, the present in vitro reaction may mimic phosphorylation of Mcm proteins during S phase. Therefore, we propose that Mcm4,6,7 helicase activity, which has functioned at the initiation of DNA replication, is inactivated by cyclin A/Cdk2 during S phase. Mcm4 protein is highly phosphorylated at G2/M phase in Xenopus (37). It has been suggested that the phosphorylation of Mcm4 prevents the binding of Mcm complex to chromatin. Although whether this phosphorylation directly affects the chromatin binding of Mcm complex remains to be determined, the phosphorylation by cyclin B/Cdk2 may play a role in preventing the initiation of DNA replication at G2/M phase.

We have shown that the DNA helicase activity is intrinsic to Mcm proteins, because site-directed mutagenesis of ATP-binding site of Mcm proteins abolished DNA helicase activity (43). Furthermore, we suggested that an ATP-binding site in Mcm4 contributes to the DNA helicase activity through the binding with single-stranded DNA, and the site in Mcm6 plays a role in high affinity binding with ATP. We examined the change in single-stranded DNA binding activity of the Mcm4,6,7 complex after phosphorylation with cyclin A/Cdk2 (Fig. 2E). The results showed that the phosphorylated Mcm4,6,7 complex, which migrated more slowly than the original complex on native gel, exhibited a reduced level of single-stranded DNA binding activity. The Mcm4,6,7 complexes containing Mcm4 deleted at amino-terminal region (Fig. 5) also showed the reduced level of the single-stranded DNA binding activity and almost no DNA helicase activity (data not shown). Thus, these results suggest that the amino-terminal region of Mcm4 plays a role in single-stranded DNA binding ability of the Mcm4,6,7 complex, and phosphorylation with Cdk2 of Mcm4 in this region affects the single-stranded DNA binding activity. Because the inhibition of Mcm4,6,7 helicase activity was detected at earlier stages of Cdk2 phosphorylation (Fig. 2, A and B), however, the effect of phosphorylation on the single-stranded DNA binding as well as the structural change of the Mcm4,6,7 complex (Fig. 2C) may not be directly linked to the inhibition of the helicase activity.

Cdc6 protein is required for the loading of Mcm proteins onto chromatin and the replication origin in S. cerevisiae (3–7). In Saccharomyces pombe, overproduction of Cdc18 protein (Cdc6 homolog) can induce over-replication (45), indicating that Cdc18 plays a critical role in determining the initiation of DNA replication. The phosphorylation of Cdc18 protein by Cdk induces the degradation of Cdc18 protein in S. pombe (46). Consistently, it has been reported that Cdc-dependent kinases prevent the formation of the prereplication complex in S. cerevisiae (25, 47). In mammalian cells, Cdc6 protein is excluded from nuclei by the phosphorylation with cyclin A/Cdk2 complex (48, 49). Thus, Cdk2 plays a critical role in the regulation of DNA replication by inactivating the function of Cdc6. However, Pianta et al. (47) have reported that forced production of Cdc6 protein after late G1 phase no longer promotes DNA replication in S. cerevisiae. These findings suggest the presence of other target(s) of cyclin-dependent kinases that play an essential role in preventing over-replication. It has been shown that Cdk2 can inhibit loading of DNA polymerase α onto chromatin in S. cerevisiae (50); this may be another reaction in the regulation of DNA replication by Cdk2. These results suggest that the cyclin-
dependent kinases phosphorylate several replication proteins including Mcm4 protein during S and G2/M phase to ensure a single round of DNA replication per cell cycle.

Acknowledgments—We thank R. A. Laskey for providing recombinant baculoviruses for preparing cyclin-dependent kinases and H. Kimura for providing anti-Mcm4 antibodies.

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