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Biochemical Properties of the Minichromosomal Maintenance Complex after the Phosphorylation by Cdc7 Kinase

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Abstract: Previous studies showed that Cdc7 kinase of *Schizosaccharomyces pombe* phosphorylated the minichromosome maintenance (Mcm) complex efficiently in the presence of spMcm10 protein. The biochemical properties of the phosphorylated Mcm complexes were examined to understand the activation mechanism of the Mcm complex by Cdc7 kinase. The phosphorylation of Mcm complex in the presence of spMcm10 by Cdc7 kinase did not affect the stability of the Mcm complex containing all six subunits, and the changes in the sedimentation properties were not observed after the phosphorylation. The reconstitution of the Mcm complex using the purified proteins showed that the phosphorylation of Mcm2 proteins did not affect the interactions between Mcm proteins. The phosphorylation of the Mcm2-7 complex at the same condition also did not activate the other biochemical activities such as DNA helicase and single stranded (ss) DNA binding activities. On the other hand, spMcm10 protein that was used for the stimulation of Mcm phosphorylation showed single stranded DNA binding activity, and inhibited the DNA helicase activity of the Mcm4/6/7 complex. These inhibitory effects were reduced by the addition of Cdc7 kinase, suggesting that the phosphorylation by Cdc7 kinase decreased the interactions between spMcm10 and the Mcm complex. Taken together, these results suggested that the phosphorylation by Cdc7 kinase alone is not sufficient for the remodeling and the activation of the Mcm complex, and the additional factors or the phosphorylations might be required for the activation of the Mcm complex.

Key words: Cdc7 kinase, DNA replication, Mcm complex, ORC, pre-RC

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The initiation of eukaryotic DNA replication is a multistep process that is regulated by a series of phosphorylation reactions (Bell and Dutta, 2002; Kelly and Brown, 2000). During G1 phase of the cell cycle, Cdc6/Cdc18, Cdt1, and minichromosome maintenance (Mcm) complex are recruited sequentially onto origin DNA in an origin recognition complex (ORC) dependent manner to form pre-replicative complex (pre-RC). Upon entering S phase, replication is initiated by the activation of pre-RC by two S phase promoting kinases, S-phase cyclin dependent kinase (S-CDK) and Cdc7 kinase. The actions of these two kinases lead to the loading of Cdc45, the unwinding of replication origins by the activation of DNA helicase activity, and the assembly of the replication fork machinery including RPA and DNA polymerases. These two kinases carry out their roles presumably by phosphorylating the components of pre-RC or the other proteins involved in the initiation of DNA replication. Although the cellular targets of the S-CDK for the replication initiation is not clear until now, the Mcm complex appears to be a major target of the Cdc7 kinase (Masai and Arai, 2002).

The Mcm complex consists of six subunits, Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7, which are all structurally related and highly conserved in eukaryotes (Tye, 1999). All of these proteins are essential for the assembly of the pre-RC, and the protein complex containing all six Mcm proteins was identified as a factor (a licensing factor) required for one round of DNA replication in the *Xenopus* system (Thommes et al., 1997). All six subunits of Mcm complex contain sequence motifs required for DNA helicase activity, and these proteins can form stable complexes containing all six subunits and the various subcomplexes such as Mcm4/6/7, Mcm 2/4/6/7, and Mcm 3/5. From these complexes, only the hetero-hexameric complex containing Mcm4, Mcm6, and Mcm7

showed DNA helicase activity *in vitro* (Ishimi, 1997; Lee and Hurwitz, 2000; Lee and Hurwitz, 2001; You et al., 2003). *In vivo* crosslinking and chromatin immunoprecipitation experiments showed that the localization of the Mcm proteins shifted from origin regions to inter origin regions during S phase (Aparicio et al., 1997). Furthermore, studies in *S. cerevisiae* with mcm degron mutants showed that the Mcm proteins are also required for the progression of the replication fork (Labib et al., 2000). Taken together, all these results suggested that the Mcm complex plays a role as the replicative DNA helicase. The Mcm complex containing all six subunits, however, did not show any significant biochemical activities *in vitro* such as DNA helicase, DNA binding, and ATPase activities (Lee and Hurwitz, 2000).

Several biochemical and genetic evidences suggested that Mcm complex is the major target of the Cdc7 kinase. Mcm proteins interact genetically and physically with Cdc7-Dbf4 kinase complex, and Mcm2 protein is a good substrate of this kinase *in vitro* and *in vivo* (Kihara et al., 2000; Lei et al., 1997; Masai et al., 2000). Furthermore, cells harboring a mutant allele of *mcm5*, *mcm5-bob1*, bypass the requirements for Cdc7 and Dbf4, suggesting that the alteration in Mcm5 satisfies the essential function of Cdc7-Dbf4 (Hardy et al., 1997). The phosphorylation of the Mcm complex containing all six subunits by Cdc7 kinase might lead to the remodeling of this complex, resulting in the activation of DNA helicase activity.

Although there were many reports suggesting that Mcm complex is the major trarget of Cdc7 kinase, the studies on the phosphorylation of Mcm complex containing all six subunits were very limited. In *Schizosaccharomyces pombe* (*S. pombe*), Cdc7 kinase (Hsk1-Dfp1) phosphorylates Mcm2-7 complex efficiently in the presence of spMcm10 (Cdc23) protein, another essential initiation factor for DNA replication, and Mcm2 and Mcm4 proteins of the complex were phosphorylated in this condition (Lee et al., 2003). However, the biological consequences of these phosphorylations were not determined yet. In order to understand the role of the Mcm complex phosphorylation by Cdc7 kinase, we have examined the biochemical properties of Mcm complex after Cdc7 phosphorylation. The analyses of DNA helicase activity, DNA binding activity and the stability of Mcm complex showed that the phosphorylation alone is not sufficient to change the biochemical properties of Mcm complex.

MATERIALS AND METHODS

Preparation of *S. pombe* Cdc7 kinase, Mcm, and Cdc23 proteins

S. pombe Cdc7 kinase complex (Hsk1-Dfp1) from Sf9 cells and Cdc23 protein from *E. coli* cells were expressed and purified as described (Lee et al., 2003). Mcm complex containing all six subunits and various Mcm subcomplex proteins were expressed and purified from Sf9 cells as described (Lee and Hurwitz, 2000).

Kinase assay

Reaction mixtures contained 25 mM Hepes-NaOH (pH 7.5), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 0.1 mM ATP, 0.1 mM sodium vanadate, and indicated levels of substrates and kinase. After incubation at 30 °C for 20 min, reaction products were analyzed by SDS-PAGE or used for further experiments.

Glycerol gradient sedimentation analysis

Protein samples were applied onto a 5 ml 15-35% glycerol gradient in buffer H (20 mM Hepes-NaOH, pH 7.5, 200 mM sodium glutamate, 2 mM magnesium acetate, 1 mM DTT, 0.05% Nonidet P-40). After centrifugation at 45,000 rpm for 13 h in a Beckman SW50.1 rotor at 4°C, fractions were collected from the bottom of the tube. The distribution of the Mcm protein was determined by SDS-PAGE and staining with silver.

DNA helicase activity assay

DNA helicase activity was measured in a reaction mixture (15 μl) containing 25 mM Hepes-NaOH (pH 7.5), 25 mM sodium acetate, 12.5 mM magnesium acetate, 4 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, 5 fmol of ^{32}P-labeled substrate (4,000 cpm/fmol), and enzyme fraction. After incubation at 32°C for 1 h, 4 μl of 5× loading buffer (100 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, and 25% glycerol) were added, and 7 μl aliquots were electrophoresed for 1 h at 150 V in a 15% polyacrylamide gel in 1× TBE (90 mM Tris, 90 mM boric acid, 1 mM EDTA). For the preparation of substrates, a 17-mer oligonucleotide (-40 sequencing primer for M13) was synthesized and annealed to M13mp18 ssDNA. After labeling of the 3'-end of the annealed DNA with [α-^{32}P]dCTP and the Klenow fragment, the labeled 18-mer/M13mp18 ssDNA was purified by Sephadex G-50 column chromatography.

Gel mobility shift assay

The 41-mer oligonucleotide (5-AATCATAGATAGTATCT CCGTGCAAGATAATCACGAGTATC-3) was labeled at the 5'-end with ^{32}P by using [γ-^{32}P]ATP and T4 polynucleotide kinase, and used as the substrate for gel mobility shift assays. Enzyme fractions were incubated at 25°C for 20 min in reaction mixtures (15 μl) containing 25 mM Hepes-NaOH (pH 7.5), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA and 20 fmol of [^{32}P]-labeled 41-mer ssDNA (4,000 cpm/fmol), in the presence

or absence of ATP or ATP analogues. After addition of 2 μl of 50% glycerol, aliquots of the reaction mixtures were electrophoresed for 3 hr at 120 V through a 4.5% polyacrylamide gel containing 6 mM magnesium acetate and 5% glycerol in 0.5× TBE at 4°C.

RESULTS

Stability of Mcm complexes after phosphorylation by Cdc7 kinase

Previous studies showed that the Mcm complex containing all six subunits (Mcm2-7) was efficiently phosphorylated by Cdc7 kinase in the presence of spMcm10 protein in *S. pombe* system (Lee et al., 2003). Since only Mcm4/6/7 complex showed detectable biochemical activities such as DNA helicase and DNA binding activities, we examined whether the phosphorylation of the six subunit Mcm complex by Cdc7 kinase lead to the dissociation of Mcm complex. For this purpose, Mcm complex was phosphorylated by the Cdc7 kinase in the presence of spMcm10 protein, and the sedimentation properties of this complex were analyzed in a glycerol gradient. As shown in Fig. 1, the sedimentation properties of the phosphorylated Mcm complex were identical to those of mock-treated Mcm complex, and the peak fractions around fraction 5 and 6 contained all six Mcm subunits. This same sedimentation property of the phosphorylated Mcm complex was not due to the lack of efficient phosphorylation, because the majority of the Mcm2 proteins in the peak fractions were the fast migrating, phosphorylated forms. This result suggested that the phosphorylation of Mcm complex by Cdc7 kinase did not lead to the dissociation or the significant conformational changes of the complex.

Although the phosphorylation of Mcm complex did not lead to the dissociation of the complex, the phosphorylation of Mcm proteins might affect the interactions between Mcm proteins. Since complete Mcm complex containing all six subunits or Mcm2/4/6/7 complex could be made using the purified Mcm4/6/7, Mcm3/5, and Mcm2 proteins, the reconstitution of Mcm complex were examined using the phosphorylated Mcm2 proteins. For this purpose, Mcm subcomplexes were mixed with Mcm2 or phosphorylated Mcm2 proteins as indicated, and the interactions between these proteins were analyzed using glycerol gradient sedimentation. As shown in Fig. 2, the incubation of phosphorylated Mcm2 protein with Mcm4/6/7 complex resulted in the dissociation of dimeric complex of Mcm4/6/7 and the formation of heterotetrameric complex of Mcm2/4/6/7. The incubation of phosphorylated Mcm2 protein with Mcm3/5 and Mcm4/6/7 also resulted in the formation of heterohexameric complex of Mcm2/3/4/5/6/7. These interaction properties of phosphorylated Mcm2 proteins were identical to those of unphosphorylated Mcm proteins

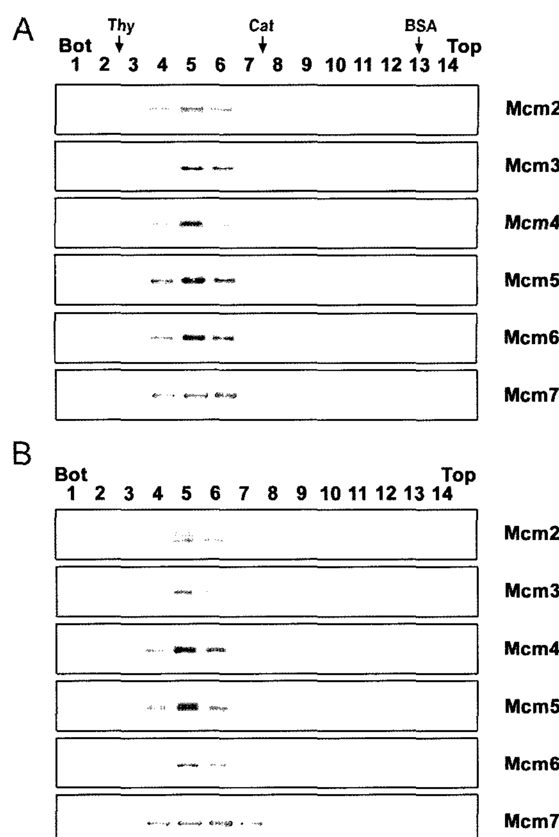

Fig. 1. Glycerol gradient sedimentation analysis of the phosphorylated Mcm complex. 10 μg of Mcm2-7 complex was phosphorylated with 1 μg of Cdc7 kinase in the presence of 10 μg of spMcm10 protein. After incubation at 30°C for 20 min, the phosphorylated (B) or mock-treated (A) Mcm complexes were loaded onto 5 ml 15-35% glycerol gradients. After centrifugation at 45,000 rpm in a Beckman SW50.1 rotor at 4°C for 13 hr, 15 fractions were collected from the bottom, and the aliquots were analyzed by Western blotting using the antibodies against the individual subunits of Mcm proteins. The marker proteins used in these experiments were thyroglobulin (Thy, 19.0 S), catalase (Cat, 11.3 S), and bovine serum albumin (BSA, 4.3 S).

(Fig. 2, left panel), suggesting that the phosphorylation of Mcm2 protein alone do not significantly affect the interactions with other Mcm subunits.

DNA helicase activity and single stranded DNA binding activity of phosphorylated Mcm complex

Although the phosphorylation of Mcm complex did not resulted in the dissociation or the significant conformational changes of the complex, it is still possible that the biochemical properties of the complex, such as DNA helicase or ssDNA binding activities might be changed by the phosphorylation. To test this possibility, the Mcm complex containing all six subunits were phsophorylated by Cdc7 kinase in the presence or the absence of spMcm10 protein, and the DNA helicase activities of these complexes were examined. As shown in Fig. 3, DNA helicase activity was not detected from the Mcm complexes that were phosphorylated by

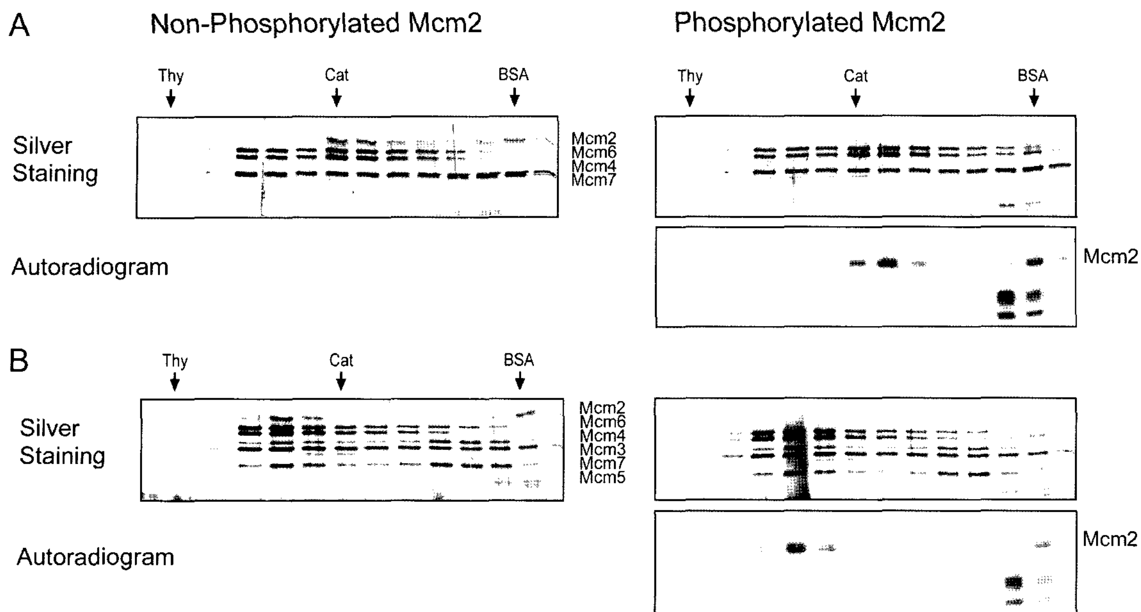

Fig. 2. Interactions between Mcm subcomplexes after the phosphorylation of Mcm2 protein by Cdc7 kinase. Mcm2 proteins (1 μg) were phosphorylated with Cdc7 kinase (0.25 μg) in the presence of [γ-^{32}P]ATP at 30°C for 20 min, and these proteins or unphosphorylated Mcm2 proteins were mixed with Mcm4/6/7, each 6 μg (A), or Mcm4/6/7 (6 μg)/3/5 (4 μg) (B) proteins as indicated in buffer T containing 0.2 M sodium glutamate. After incubation at 25°C for 20 min, glycerol gradients sedimentation analysis was performed. Fractions were analyzed by SDS-PAGE followed by staining with silver or autoradiography.

Cdc7 kinase, suggesting that the phosphorylation alone may not sufficient to activate the DNA helicase activity of Mcm complex. During the course of these experiments, however, we found that spMcm10 protein inhibit the DNA helicase activities of Mcm4/6/7 (Fig. 4). The addition of increasing amount of spMcm10 protein in the reactions completely knocked down the DNA helicase activity of Mcm4/6/7 (Fig. 4, lanes 2~5), and the addition of Cdc7 reduced the inhibitory effects of spMcm10 (Fig. 4, lanes 11~14). DNA helicase activity of Mcm4/6/7 complex completely inhibited at the level of spMcm10 that is required for the stimulation of the efficient phosphorylation of the Mcm complex. Since the efficient phosphorylation of Mcm complex only occurred in the presence of spMcm10 protein and the DNA helicase activities were inhibited by the presence of spMcm10 protein, DNA helicase activities of the Mcm compelx could not be detected at this condition, even if there is the activation of DNA helicase activity by the phosphorylation. Therefore, we could not rule out the possibility that the phosphorylation of the Mcm complex by Cdc7 kinase still activates the DNA helicase activity of this complex.

Then we examined the ssDNA binding acitivity of the Mcm complex after Cdc7 phosphorylation. If there is the activation of DNA helicase activity by the phosphorylation, the Mcm complex must contain ssDNA binding activity after Cdc7 phosphorylation. When we incubated 41-mer

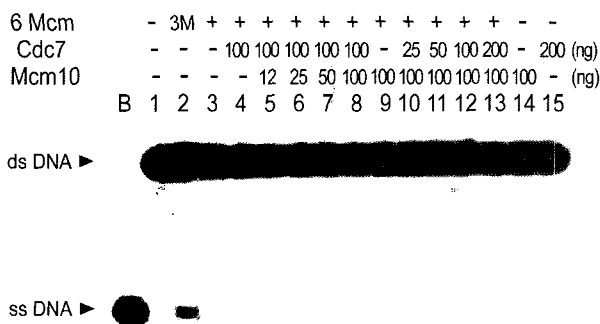

Fig. 3. DNA helicase activity of Mcm2-7 complex after the phosphorylation by Cdc7 kinase. Mcm proteins (0.7 μg) were phosphorylated with various levels of Cdc7 kinases in the presence or the absence of Cdc23 proteins as indicated. After incubation at 30°C for 20 min, DNA helicase activities of the reaction mixtures were analyzed as described in Materials and Methods.

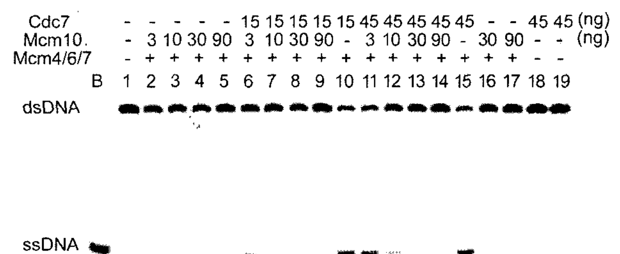

Fig. 4. DNA helicase activity of Mcm4/6/7 complex was inhibited by spMcm10 protein. Mcm 4/6/7 proteins were incubated with Cdc7 kinase and spMcm10 as indicated. After incubation at 30°C for 20 min, DNA helicase activity assays were carried out by the addition of Mcm/ssDNA substrates, and further incubation for 1 hr.

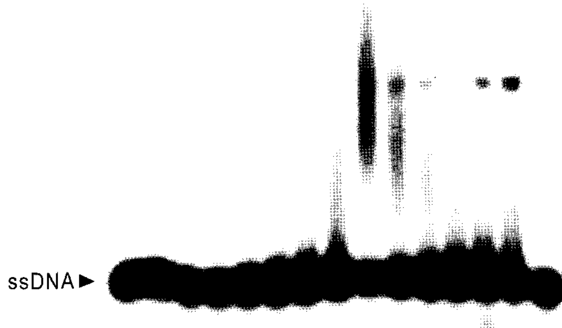

Fig. 5. Single stranded DNA binding activity of Mcm2-7 complex after phosphorylation by Cdc7 kinase. Mcm proteins (0.7 μg) were phosphorylated with Cdc7 kinases in the presence or the absence of Cdc23 proteins as indicated. After incubation at 30°C for 20 min, ssDNA binding activities of the reaction mixtures were analyzed as described in Materials and Methods.

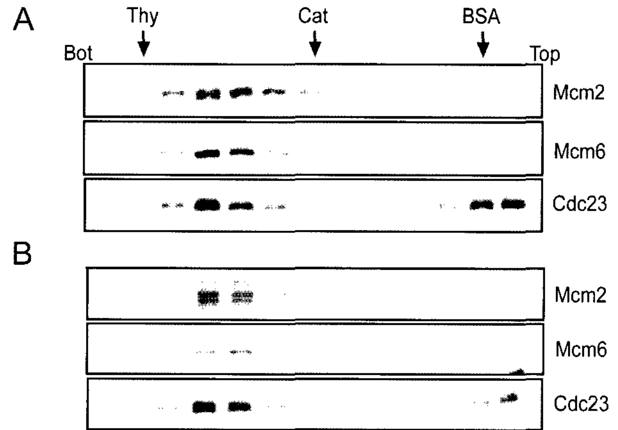

Fig. 6. Interactions between Mcm2-7 complex and spMcm10 protein after the phosphorylation by Cdc7 kinase. Mcm2-7 complex (10 μg) and spMcm10 proteins were mixed in a kinase reaction mixture, and incubated in the presence (B) or the absence (A) of Cdc7 kinase. After incubation at 30°C for 20 min, 15-35% glycerol gradient sedimentation analysis was performed, and the fractions were analyzed by Western blotting.

single stranded DNA with the Mcm complex in the presence or the absence of Cdc7 kinase and spMcm10 protein, we detected the shifted bands in the lanes containing spMcm10 proteins. These shifted bands appeared in the lane containing spMcm10 protein alone (Fig. 5, lane 14), and the additions of Mcm complexes with spMcm10 lead to the supershift of this band (Fig. 5, lanes 5~14), suggesting the interaction between the Mcm complex and spMcm10 proteins. Except for those bands, we did not detect the bands specific for the Mcm complex, suggesting that the phosphorylation of the Mcm complex alone is not sufficient to lead to the activation of the biochemical properties such DNA helicase or DNA binding activities. Interestingly, the additions of the increasing amounts of Cdc7 kinase decreased the amount of the supershifted complex containing the Mcm complex and spMcm10 protein (Fig. 5 lanes 10~13). These results suggested that the phosphorylation of the Mcm complex and/or spMcm10 protein by Cdc7 kinase affect the interactions between these proteins. However, when we examined the interactions between spMcm10 protein and Mcm complex using glycerol gradient sedimentation analysis, stable interactions were detected even after the phosphorylation of proteins with Cdc7 kinase (Fig. 6). These results suggested that the phosphorylation by Cdc7 kinase affected the interactions between Mcm complex and spMcm10 protein, but the phosphorylation alone is not enough to prevent the association of these proteins at this condition.

DISCUSSION

Cdc7 is an essential kinase for the initiation of eukaryotic DNA replication, whose activity is required for the firing of individual replication origin. Several genetic and biochemical evidences suggested that Mcm complex is a major target of Cdc7 kinase (Masai and Arai, 2002). Since Mcm complex is a major candidate of replicative helicase, which is responsible for the unwinding of replication origin and the elongation of replication fork, it is very attractive model that the phosphorylation of Mcm complex by Cdc7 kinase directly activates the DNA helicase activity. The Mcm complex containing all six subunits did not show any detectable DNA helicase activity (Lee and Hurwitz, 2000). On the other hand, Mcm4/6/7 subcomplex has shown to be a processive DNA helicase (Lee et al., 2003; You et al., 2003). The phosphorylation of Mcm complex might lead to the activation of DNA helicase by remodeling the complex by the conformational changes itself or the dissociation of the complex into active subcomplex of Mcm4/6/7.

When the stabilities and the biochemical properties of the Mcm complex were examined, we failed to detect the DNA helicase activity from the Mcm complex phosphorylated by Cdc7 kinase. We also did not see any dissociation of the Mcm complex to the subcomplexes after phosphorylation. The pre-RC formed at G1/S transition stages not only contains Mcm complexes, but also contains several other factors essential for the initiation of DNA replication. Cdc7 kinase also is the components of the pre-RC. Additional factors or the phosphorylation of these proteins might be

required for the activation of DNA helicase activities. Cdc45 protein appeared to be a good candidate because this protein is known to interact with Mcm complex and is essential for origin unwinding. The interactions between the components of the pre-RC or the proper tertiary structure of the complex might be also required for the activation of Mcm complex. *In vitro* phosphorylation analysis reported previously showed that many proteins involved in DNA replication such as Cdc45, and polymerase α were phosphorylated by Cdc7 family kinase (Weinreich and Stillman, 1999). During this study, we also detected the efficient phosphorylation of Orc4 protein in ORC and spMcm10 protein (data not presented). Although the biological roles of these phosphorylations are not clear until now, those phosphorylations might also contribute to the remodeling and activation of pre-RC. However, we still could not rule out the possibility that the phosphorylation of the Mcm complex by Cdc7 kinase directly lead to the activation of the DNA helicase activity of Mcm complex. In this study, spMcm10 proteins were used for the phosphorylation of Mcm complex by Cdc7 kinase. In the absence of spMcm10, Cdc7 kinase did not efficiently phosphorylate the Mcm complex. Since spMcm10 protein contains intrinsic DNA binding activity and inhibits the DNA helicase activity of the Mcm4/6/7 complex, DNA helicase activity can not be detected at this condition, even if there is any activation by Cdc7 phosphorylation. Other phosphorylation systems, such as the phosphorylation reactions using the pre-RC as a substrate, are required to obtain the more direct evidence of the biochemical effects of the phosphorylation.

In this study, we found that spMcm10 stably interacted with the Mcm complex, and the phosphorylation of these proteins with Cdc7 kinase slightly affected the interactions between these proteins. We also found that the inhibitory effects of spMcm10 protein to the Mcm4/6/7 DNA helicase activity decreased after the phosphorylation by Cdc7 kinase. Although the biochemical roles of spMcm10 protein during the initiation processes is not clear until now, these results and the interactions of spMcm10 protein with many other initiation factors suggested that spMcm10 protein might be essential for the activation process of the Mcm complex by Cdc7 kinase.

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