Potent DNA strand annealing activity associated with mouse Mcm2~7 heterohexameric complex

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ABSTRACT

Mini-chromosome maintenance (Mcm) is a central component for DNA unwinding reaction during eukaryotic DNA replication. Mcm2~7, each containing a conserved ATPase motif, form a six subunit-heterohexamer. Although the reconstituted Mcm2~7–Cdc45–GINS (CMG) complex displays DNA unwinding activity, the Mcm2~7 complex does not generally exhibit helicase activity under a normal assay condition. We detected a strong DNA strand annealing activity in the purified mouse Mcm2~7 heterohexamer, which promotes rapid reassociation of displaced complementary single-stranded DNAs, suggesting a potential cause for its inability to exhibit DNA helicase activity. Indeed, DNA unwinding activity of Mcm2~7 could be detected in the presence of a single-stranded DNA that is complementary to the displaced strand, which would prevent its reannealing to the template. ATPase-deficient mutations in Mcm2, 4, 5 and 6 subunits inactivated the annealing activity, while those in Mcm2 and 5 subunits alone did not. The annealing activity of Mcm2~7 does not require Mg^{2+} and ATP, and is adversely inhibited by the presence of high concentration of Mg^{2+} and ATP while activated by similar concentrations of ADP. Our findings show that the DNA helicase activity of Mcm2~7 may be masked by its unexpectedly strong annealing activity, and suggest potential physiological roles of strand annealing activity of Mcm during replication stress responses.

INTRODUCTION

To ensure once and only once replication of the genome during cell cycle, the Mcm (mini-chromosome maintenance) 2~7 complex is loaded onto origins through origin recognition complex (ORC), Cdc6 and Cdt1, establishing pre-replicative complexes (1,2). The Mcm complex is a central component for DNA unwinding reaction during eukaryotic DNA replication. It is composed of six subunits containing conserved ATPase motifs. They exist in multiple forms. The Mcm2~7 heterohexamer is the core of replicative helicase, that transforms into Mcm2~7 double-hexamer and then Cdc45–Mcm2~7–GINS (CMG) complex during the assembly of the replication fork (3,4). Initially, a Mcm4/6/7 subcomplex was shown to possess DNA helicase activity (5,6). It was also shown that Mcm2 inhibits the helicase activity of Mcm4/6/7 by disrupting the hexamer structure of Mcm4/6/7 and forming Mcm2/4/6/7 complex (6,7). A similar activity was also reported for the Mcm3/5 complex (7). *In vivo* observations implicate the Mcm2~7 complex as the replicative helicase (2). However, DNA helicase activity has not been detected in the purified heterohexameric complex of Mcm2~7. It was reported that the *Saccharomyces cerevisiae* Mcm2~7 possesses weak helicase activity in the presence of high levels of acetate or glutamate ions (8). However, we and other groups failed to detect the helicase activity with the human, Drosophila, fission yeast, and mouse Mcm2~7 complexes under the similar reaction conditions (9–11).

Accumulating evidence indicates that Cdc7 kinase (also known as Dbf4-dependent kinase, DDK) and cyclin-dependent kinase (CDK) are required for helicase activation (3,4). Cdc45 is recruited to Mcm in a Cdc7-dependent manner, and the GINS complex is recruited in a Cdc7 and CDK-dependent manner (12,13). Indeed, the interaction of Mcm2~7 complex with Cdc45 and GINS create the CMG complex, that is active in DNA unwinding (10,14). DNA helicase activity of Mcm may be regulated also by modification of the protein. It is now known that Mcm2, Mcm4 and Mcm6 are the key substrates of Cdc7 and CDK (15–21). *In vitro*, phosphorylation of the Mcm4/6/7 complex results in decreased helicase activity (22–25). In addition to phosphorylation, the Mcm2~7 SUMOylation counteracts the phosphorylation and ensures precise replication initiation (26). SUMOylation is also known to affect genome maintenance and to play key roles in response to DNA damage (27). Recent works show that Mcm is recruited to the sites of DNA damage and colocalizes with γ-H2A (28). At stalled replication forks, fork regression may occur to promote repair of lesions responsible of fork stall. During this process, displaced double-strand DNA needs to be reannealed. In fact, DNA strand annealing activity has been discovered in vari-

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ous helicases including RecQ (29–31), Pif1 (32); Dna2 (33), TWINKLE mitochondrial DNA helicase (34), and annealing helicases HARP and AH2 (35–37).

Here, we show that the recombinant mouse Mcm2∼7 complex exhibits potent DNA helicase activity in the presence of a trap DNA that hybridizes with the displaced single-stranded DNA (ssDNA). We further discovered that the Mcm2∼7 possesses DNA annealing activity that competes with DNA unwinding activity. Through comparison of the wild type and mutated Mcm2∼7 complexes, we have systematically characterized the DNA helicase, strand-annealing, DNA binding, and ATP hydrolysis activities and showed that the DNA strand annealing activity is intrinsic to Mcm2∼7 and that the conserved Walker and Arginine finger domains are essential for the annealing activity. The results presented in this communication suggest that the Mcm2∼7 complex has an intrinsic DNA helicase activity, which is normally masked by its strong strand annealing activity under normal assay conditions. We will discuss potential role of DNA annealing activity of Mcm in processing of stalled replication forks.

MATERIALS AND METHODS

DNA substrates

All DNA substrates were prepared by annealing appropriate oligonucleotides together and were purified with Sep-Pak C18 cartridge chromatography (Waters) after gel electrophoresis on native PAGE. Y-fork substrates were prepared by annealing a 5′-end-labeled oligonucleotide, dT_{20}-25R (5′-dT_{20}-CGACGTTGTAAAACGAGCCCGAGTG-3′) or dT_{20}-37R (5′-dT_{20}-ACTGGCCGTCGTTTTACA ACGTCGTGACTGGGAAAAC-3′), with an unlabeled 50mer-dT_{60} (38) or 37mer-dT_{40} (38), as indicated.

Expression and purification of wild-type and mutated Mcm2–7 proteins

All of the proteins were expressed using the baculovirus system and were purified from baculovirus-infected insect cells. The purification of the wild-type Mcm2∼7, Mcm4/6/7, Mcm2/4/6/7, Mcm3/5 complexes was conducted as previously (39–41), with several additional steps. The Mcm2∼7 complex was reconstituted from the purified Mcm2/4/6/7 and Mcm3/5 complexes which had been generated by cotransfection of His_6Mcm2–7 + His_6Mcm4–6 viruses and transfection of Mcm3–5His_6-Flag viruses, respectively. Equimolar amounts of Mcm2/4/6/7 and Mcm3/5, purified through Ni-NTA-agarose alone and the combination of Ni-NTA-agarose and anti-Flag M2 antibody–agarose affinity chromatography steps, respectively, were mixed and centrifugation at 36,000 rpm for 17 h (Beckman, TLS55 rotor) on a 15–35% liner glycerol gradient. The peak fractions were collected and injected into a MonoQ column connected to the SMART system (GE Healthcare Life Sciences). The column was equilibrated in buffer (20 mM HEPES–NaOH (pH 7.5), 1 mM DTT, 1 mM EDTA, 0.01% Triton X-100, 10% glycerol and protease inhibitors) containing 0.3 M potassium-acetate and developed with 1 ml of 0.3–1.2 M K-acetate gradient. The peak fractions

of Mcm2∼7 complex, eluted around 0.85 M potassium-acetate, were pooled and then were fractionated on second glycerol gradient in buffer containing 20 mM HEPES–NaOH (pH 7.5), 0.15 M potassium acetate, 1 mM DTT, 1 mM EDTA, 0.01% Triton X-100 and 1 mM PMSF.

The same protocol was used to purify mutant Mcm2∼7 complexes with the following modification. Site-directed mutagenesis of the Mcm2 and Mcm5 was conducted using QuikChange Site-directed Mutagenesis Kit (Agilent Technologies). The oligonucleotides 5′-GAGCCCATCATTTC CGCCTTTGATGTCCTGTGTG-3′ and 5′- CACACA GGACATCAAAGGCGGAAATGATGGGCTC-3′ were used as primers to introduce Arg to Ala mutation at the Arginine finger motif of Mcm2. The oligonucleotides 5′- ACCCTGGTACAGCCGCGGCTCAGCTT CTGAAG-3′ and 5′-CTTCAGAAGCTGAGCCGCGG CTGTACCAGGGT-3′ were used as primers to introduce Lys/ Ser to Ala/ Ala mutation at Walker A motif of Mcm5. The mutant Mcm4DE-6DE (Asp/Glu to Ala/Ala mutation) was prepared previously (6). The mutants Mcm2∼7(2,5) containing Mcm2R-A and Mcm5KS-AA and Mcm2∼7(2,4,5,6) containing Mcm2R-A, Mcm5KS-AA, Mcm4DE-AA and Mcm6DE-AA were purified as described above for the wild-type. Mcm2-His_6 was cloned into pFastBacHTA vector, permitting the expression of a single subunit of Mcm2. The expressed Mcm2 protein in insect cells was purified by consecutive steps involving nickel column chromatography, Mono Q column chromatography (twice), and finally 15–35% glycerol gradient centrifugation at 36,000 rpm for 17 h.

DNA binding, DNA helicase and ATPase assays

DNA helicase and DNA binding activities were examined in reaction mixtures (12 μl) containing 5 mM creatine-phosphate, 20 mM Tris–HCl (pH 7.5), 10 mM Mg-actetate, 1 mM DTT, 0.1 mM EDTA, 20% glycerol, 50 μg/ml BSA and 60 mM K-glutamate in the presence of the fork DNA substrate. In the DNA helicase assay, 10 mM ATP and unlabeled trap DNA were added and incubated at 37°C for 60 min. After termination of the reaction by adding Proteinase K (4 mg/ml), SDS (0.4%) and incubation at 37°C for 15 min, the stop buffer (final 20 mM EDTA, 0.1% SDS and 0.25% BPB) was added and products were separated on 10% acrylamide/1× TBE gel. DNA binding assays were conducted at 30°C for 30 min with 1 mM ATP-γ-S replacing 10 mM ATP. All of samples were run on 5% non-denaturing gel containing 0.5× TBE, 6 mM Mg-acetate, and 5% glycerol. ATPase activities were examined with equal amount of wild type or mutants Mcm2∼7 protein in the presence of oligonucleotide DNA as previously described (42).

DNA strand annealing assays

The DNA annealing reaction was carried out at 30°C for 30 min or indicated times using 10 fmol of labeled dT_{20}-37R and 15 fmol of unlabeled 37-dT_{10} (38) oligonucleotides. Standard reaction buffer contained 5 mM creatine-phosphate, 20 mM Tris–HCl (pH 7.5), 5 mM DTT, 0.1 mM EDTA, 50 μg/ml BSA, 0.01% Triton X-100. The reaction was stopped with quenching solution (20 mM

EDTA, 0.1% SDS and 0.2% BPB). The amount of annealed fork DNA was analyzed on 10% nondenaturing PAGE/1× TBE. In some assays, magnesium acetate, ATP, ATP-γ-S and ADP were present as indicated.

The coupled reaction of phosphorylation and DNA annealing analysis in Figure 6 were conducted as follows. After phosphorylation of Mcm2∼7 with various kinases for 30 min at 30°C, a mixture were diluted into above reaction buffer and partially complementary two oligonucleotides (Y-fork; one of which was labeled) were added, and the incubation was continued for 30 min at 30°C as described above.

Kinase assays

In vitro phosphorylation of Mcm2∼7 with Cdk2/Cyclin A, Cdk2/Cyclin E, Cdc2/Cyclin B or Cdc7/ASK complex were conducted basically as described previously (43). Phosphorylation and DNA-binding were carried consecutively. The Mcm2∼7 complex was phosphorylated by various kinases, and then the effect of phosphorylation on DNA-binding activities were analyzed with gel-shift and biotin–DNA pull-down assays.

DNA-binding analyses using biotin-labeled fork–DNA

Biotin-labeled fork-DNA was made by annealing biotin-labeled 37mer-dT_{40} oligonucleotide and the unlabeled complementary dT_{20}-37R. The DNA affinity was analyzed by pull-down using biotin–streptavidin magnetic beads as described previously (43).

RESULTS

Purification of wild-type and mutant Mcm2∼7 complexes

The wild-type and two mutant forms of the Mcm2∼7 complexes were expressed in insect cells and purified with consecutive step (Figure 1A and B). Mcm2∼7(2,5) contains a mutant Mcm2 (Mcm2R) in which the arginine in the arginine finger motif is replaced with alanine and a mutant Mcm5 (Mcm5KS) in which the lysine and serine in the Walker-motif A of Mcm5 are substituted by alanine. Mutant Mcm2∼7(2,4,5,6) contains additional Walker-motif B mutations in Mcm4 (Mcm4DE) and 6 (Mcm6DE) (Figure 1A). The Mcm2/4/6/7 complex was expressed by cotransfection of the recombinant virus coexpressing Mcm2 and 6xhis-Mcm7 and that coexpressing 6xhis-Mcm4 and Mcm6, and then was partially purified by Ni-NTA agarose. The recombinant virus coexpressing Mcm3 and 6xhis-flag-Mcm5 was used to express the Mcm3/5 complex, which was purified by consecutive steps of Ni-NTA agarose and anti-Flag M2 affinity column. To obtain the stoichiometric Mcm2∼7 heterohexameric complex, we pooled the partially purified Mcm2/4/6/7 and Mcm3/5 in an optimum ratio, and then centrifuged the mixture in 15–35% glycerol gradient. The main fractions containing the Mcm2∼7 complex were further purified by MonoQ column chromatography and the second glycerol gradient centrifugation to completely remove the nuclease contamination. The purified wild-type and two mutant Mcm2∼7 complexes contained stoichiometric amount of each subunit (Figure 1C and D). Furthermore, the wild-type and mutant complexes

migrated as ∼600 kDa hexameric complexes on a native polyacrylamide gel (Figure 1E, right). We noted that the Mcm4DE and Mcm6DE proteins migrated faster than the wild-type on SDS-PAGE (Figure 1E, left, lane 3) (6) and that the mutant Mcm2∼7 complex containing the mutation of Mcm4DE and Mcm6DA migrated slightly faster than the wild-type in native PAGE (Figure 1E, right, lane 4).

The helicase activity of Mcm2∼7 heterohexameric complex is detectable in the presence of a trap DNA

The *S. cerevisiae* Mcm2∼7 was reported to possess weak helicase activity in the presence of high levels of acetate or glutamate (8). However, helicase activity was not detected in the human, Drosophila, fission yeast, and mouse Mcm2∼7 complex under the similar conditions (Figure 2B, lanes 2–4) (9–11). It has also been known that the Mcm2∼7 forms a complex with Cdc45 and GINS to generate helicase–active CMG complex (10,14). These results indicate two possibilities. The first is that Mcm2∼7 complex is devoid of intrinsic helicase activity, but the association with Cdc45 and GINS induces the formation of an active helicase. The second is that Mcm2∼7 possesses the intrinsic DNA helicase activity which is somehow inactivated or inhibited in the heterohexamer complex, and that the inhibition is removed upon association with Cdc45 and GINS.

The Mcm2∼7 complex failed to unwind the fork substrate containing a 25 bp duplex segment, whereas Mcm4/6/7 complex efficiently unwound the same substrate (Figure 2B, compare lane 10 to lanes 2–4). However, in the presence of a 25-fold excess of oligonucleotide (dT_{20}-25R), as a trap for the displaced unlabeled strand of oligonucleotide, efficient DNA unwinding activity was detected with the Mcm2∼7 complex (Figure 2B, lanes 6–8). The profile of the helicase activity of the glycerol gradient fractions coincided with that of the hexameric Mcm2∼7 protein (Figure 3A–C). The above result led us to propose that the failure to observe Mcm2∼7 DNA unwinding activity *in vitro* could be due to its DNA annealing activity, as was reported for other helicases (31,37,44).

Mcm2∼7 complex possesses DNA strand annealing activity in the absence of cofactors

We then examined the DNA strand annealing activity of Mcm2∼7. We conducted DNA annealing assay with the glycerol gradient fractions of the Mcm2∼7 complexes that were used in DNA helicase assay in Figure 3. The DNA strand annealing activity correlated with the amount of Mcm2∼7 in glycerol gradient fractions #2 to #6 (Figure 4A). The strand annealing activity in fractions #7–#9 is due to the presence of Mcm3/5 subcomplex (see Figure 7). Strong DNA annealing activity was detected in highly purified Mcm2∼7 complex in the absence of Mg^{2+} and nucleotides (ATP) (Figure 4B, lanes 2 and 3). The strand annealing activity of Mcm2∼7 peaked in 6 min (Figure 4B, lanes 14–18). In contrast, the helicase-active Mcm4/6/7 complex exhibited very little annealing activity under the same condition (Figure 4B, lanes 4–6, and lane 19).

Mcm helicase requires Mg^{2+} and ATP to catalyze DNA unwinding. We thus tested their effects on the strand annealing activity of Mcm2∼7. Mg^{2+} at the concentration of 1
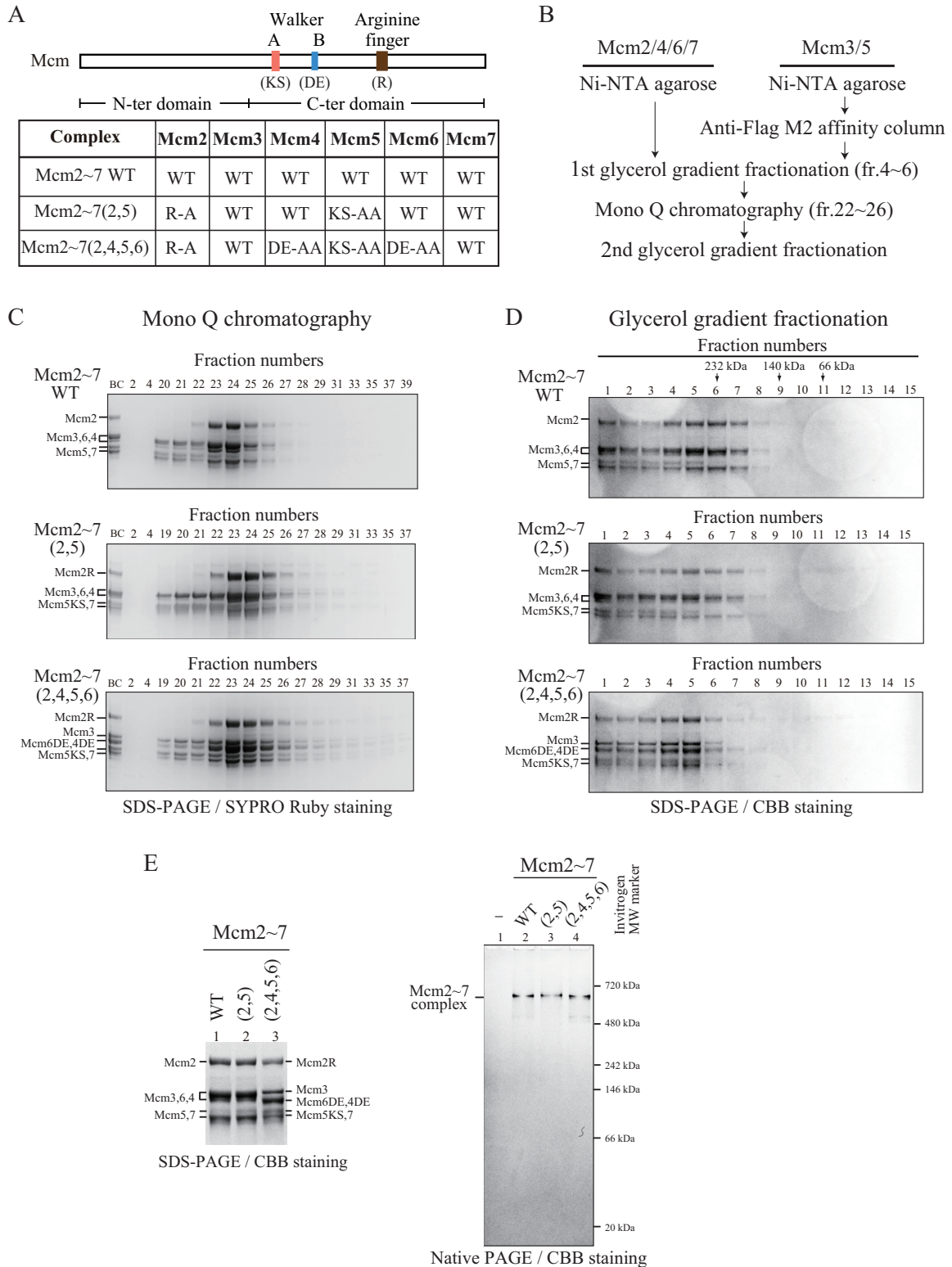
Figure 1. Purification of mouse Mcm2~7 complex. (**A**) Schematic representation of Mcm protein showing the Walker A and B, and Arginine finger motifs. Mcm2~7 complexes formed by various wild type and mutated Mcm subunits are summarized. (**B**) Diagram explaining the procedure for purification of the Mcm2~7 complex. (**C**) Wild-type or mutant forms of the Mcm2~7 complexes expressed in insect cells were purified by Ni-NTA, anti-Flag antibody beads, first glycerol gradient fractionation, and then the peak fractions (fractions 4–6) of these Mcm complexes were further purified by Mono Q chromatography. BC: before column. (**D**) The peak fractions of Mono Q chromatography (fractions 22–26) were pooled and further fractionated on second 15–35% glycerol gradient at 36,000 rpm for 17 h. Each fraction was subjected to SDS-PAGE (7.5% polyacrylamide) and stained with Coomassie Brilliant Blue (CBB). (**E**) The complex formation of the wild-type and mutant Mcm2~7 complexes were examined on a 5–20% gradient native gel (right) in parallel with the analysis on SDS-PAGE (left). The mutant Mcm2~7, like the wild type, can form heterohexameric complexes.
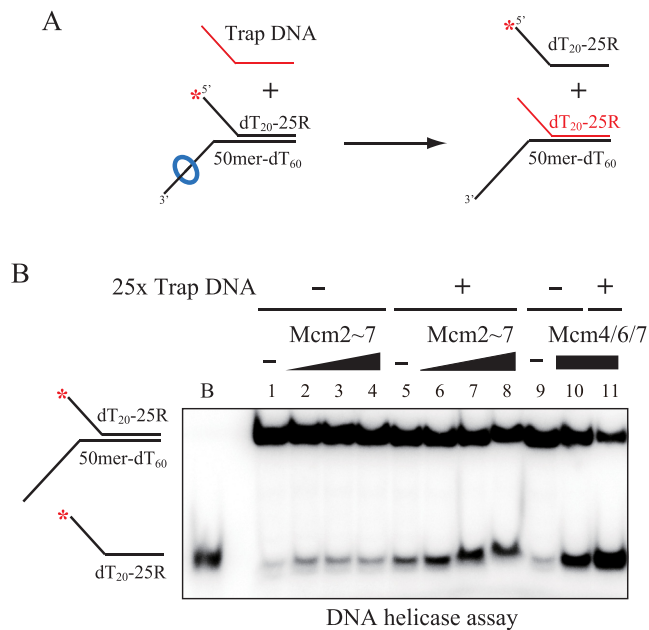
Figure 2. Effect of a 'trap' oligonucleotide on the helicase activity of Mcm2∼7. (**A**) Scheme of DNA helicase assay of Mcm2∼7 in the presence of an excess amount of unlabeled trap DNA. DNA unwinding allows the displacement of ^{32}P-labeled dT_{20}-25R (indicated with an asterisk) from the Y-fork DNA. The trap DNA anneals with and unlabeled $50mer$-dT_{60} and prevents reannealing of the displaced labeled dT_{20}-25R with $50mer$-dT_{60}, permitting the measurement of the DNA helicase activity of Mcm2∼7 (shown as a circle). (**B**) The Y-fork substrate was unwound by the Mcm2∼7 complex in the presence of 25-fold unlabeled trap oligonucleotide (the dT_{20}-25R). Reaction mixtures contained a fork DNA substrate (dT_{60}-$50mer$/dT_{20}-25R; 15 fmol) with or without the trap DNA (dT_{20}-25R; 375 fmol), Mcm2∼7 proteins (50, 100 and 200 ng) or Mcm4/6/7 (100 ng). The amount of fork DNA and unwound ssDNA was analyzed on 10% non-denaturing PAGE/1× TBE.

mM slightly stimulated the annealing activity, whereas that larger than 5 mM resulted in inhibition of the activity (Figure 4C). This result was reproducible with other batch of Mcm2∼7 protein (Supplementary Figure SA). Unexpectedly, ATP at the concentration over 2.5 mM dramatically inhibited the DNA strand annealing activity (Figure 4D and E). A non-hydrolysable ATP analog, ATP-γ-S also inhibited the annealing activity of Mcm2∼7 (Figure 4E). In contrast, the annealing activity of Mcm2∼7 was dramatically increased in the presence of ADP in a dose-dependent manner (up to 7.5 mM) (Figure 4E). The efficient unwinding activity of Mcm2∼7 needs a high concentration of ATP but is not supported by ADP (data not shown) (45). Thus, we suggest that a nucleotide cofactor bound to Mcm2∼7 protein may act as a switch to modulate the two opposing activities; ATP drives displacement of DNA, whereas ADP triggers annealing of complementary DNA strands. Similar observation was previously reported for DEAD box helicases (46,47).

To determine the role of the ssDNA and fork DNA binding of Mcm2∼7 in strand annealing, reactions were carried out in the presence of ssDNA and fork DNA competitors. The presence of a fork DNA competitor inhibited the annealing reaction by Mcm2∼7, whereas that of ssDNA had no effect (Figure 4B, lanes 8 and 11). The distinct effects of

fork DNA and ssDNA may reflect the fact that Mcm2∼7 has higher affinity to fork DNA. Mcm2∼7 can bind to Y-fork or bubble DNA more efficiently than to ssDNA (Supplementary Figure SB and data not shown). Above results suggest that the strand annealing activity of Mcm2∼7 may involve its preferential affinity to fork DNA.

Effect of ATP hydrolysis mutations on the DNA annealing activity of Mcm2∼7

Each subunit of Mcm2∼7 contains ATP binding domain, and it has been shown that mutations of the conserved ATP binding sites in each Mcm subunit lead to loss of its biological functions (48,49). We previously reported that Mcm4DE/6DE/7 complex is devoid of helicase and ATP hydrolysis activities (6). We therefore introduced similar mutations into Mcm2∼7 complex. In addition to Mcm4DE and Mcm6DE mutations, we have constructed Mcm5KS (KS in the Walker A motif) and Mcm2R (R in the arginine finger) mutations (Figure 1). Using highly purified wild-type and mutant hexameric Mcm2∼7 complexes, we carefully compared the helicase, DNA-binding, and ATPase activities. In the presence of a trap DNA, helicase activity was detected in wild-type of Mcm2∼7, but not in the two mutant forms of Mcm2∼7 complexes, Mcm2∼7(2,5) and Mcm2∼7(2,4,5,6) (Figure 5A). This result indicates that the conserved Walker motifs and Arginine finger domain are essential for DNA helicase activity of the Mcm helicase, consistent with previous reports (6,8). This result also provides strong evidence that the DNA helicase activity observed on Mcm2∼7 in the presence of a trap DNA represents the activity intrinsic to the Mcm2∼7 complex, not that due to a contaminating other forms of Mcm complexes.

We next examined the ATP hydrolysis activities by each Mcm2∼7 mutant in the presence of an oligonucleotide. We discovered that both Mcm2∼7(2,5) and Mcm2∼7(2,4,5,6) complexes display ATPase activities comparable to that by the wild type (Figure 5B). This result is similar to that of the studies on the *Sulfolobus solfataricus* Mcm hexameric complex, which showed that the mutant complex containing three ATPase-mutant subunits still displayed ATP hydrolysis activity (50). It was also reported that *S. cerevisiae* Mcm2∼7 complex with triple Walker A mutations retains the ATPase activity (48,49). Biochemistry and structure analysis of Mcm2∼7 complex has showed that Mcm3/7 dimer has an activity comparable to that of the whole Mcm2∼7 complex (51,52).

We then examined the DNA-binding activity of these mutants using the Y-fork substrates. We found that both Mcm2∼7(2,5) and Mcm2∼7(2,4,5,6) complexes show DNA binding activity comparable to that shown by the wild type Mcm2∼7 complex (Figure 5C). These results indicate that the lack of helicase activity by Mcm2∼7 is not due to the loss of overall ATP hydrolysis activity or to ineffective fork DNA binding.

We then examined the effect of these ATP binding mutations on DNA annealing activity of Mcm2∼7. Mcm2∼7(2,5) retained efficient annealing activity comparable to that by the wild type Mcm2∼7, whereas Mcm2∼7(2,4,5,6) was almost completely defective in strand annealing (Figure 5D, compare lanes 2–4, 5–7 and 8–10).
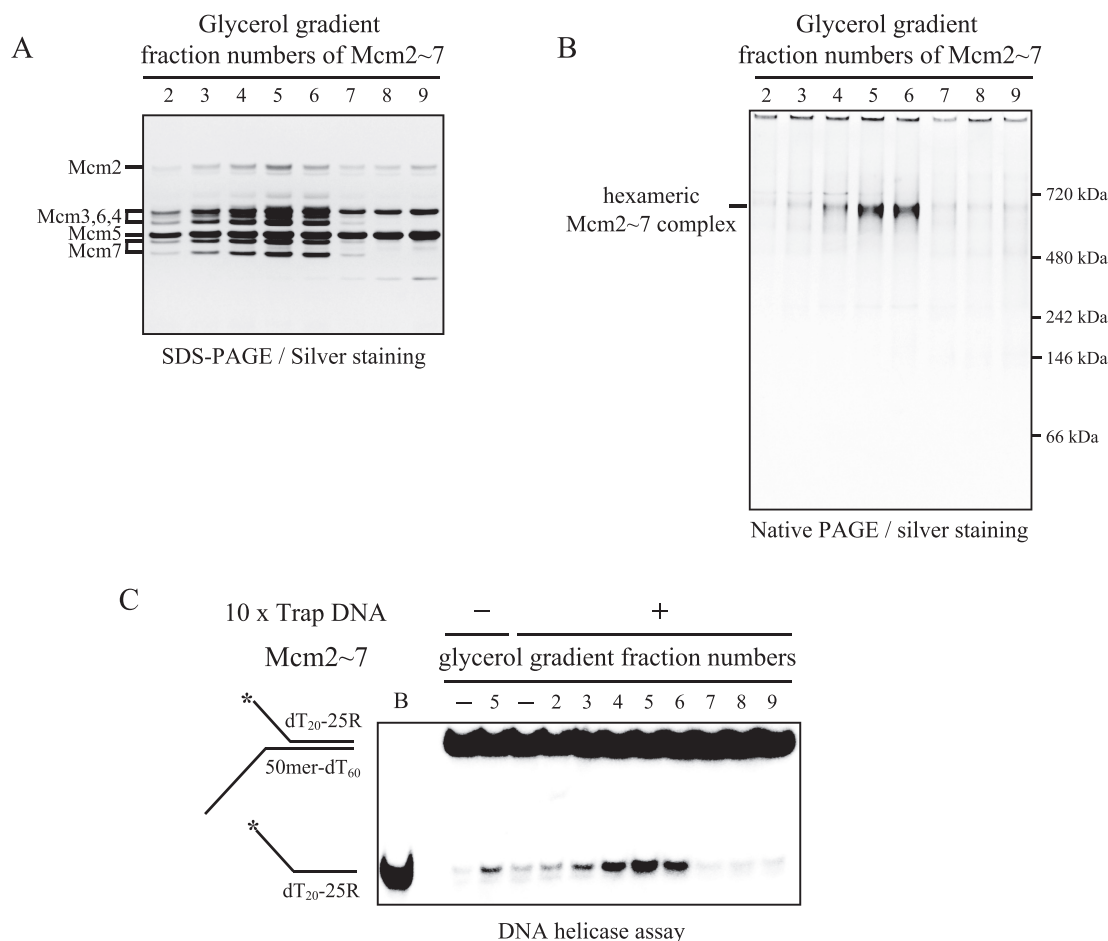
A

Glycerol gradient
fraction numbers of Mcm2~7

2 3 4 5 6 7 8 9

Mcm2

Mcm3,6,4
Mcm5
Mcm7

SDS-PAGE / Silver staining

B

Glycerol gradient
fraction numbers of Mcm2~7

2 3 4 5 6 7 8 9

hexameric
Mcm2~7 complex

720 kDa

480 kDa

242 kDa

146 kDa

66 kDa

Native PAGE / silver staining

C

10 x Trap DNA − +

Mcm2~7   glycerol gradient fraction numbers

B − 5 − 2 3 4 5 6 7 8 9

* dT$_{20}$-25R

50mer-dT$_{60}$

*

dT$_{20}$-25R

DNA helicase assay

Figure 3. DNA helicase activity of the heterohexameric Mcm2~7 complex purified on glycerol gradient. The 15–35% glycerol gradient fractions (36,000 rpm for 17 h) of Mcm2~7 were examined in SDS-PAGE (7.5% polyacrylamide) (**A**) or 5–20% gradient native gel (**B**) and stained with silver. (**C**) DNA helicase activity of Mcm2~7 was examined in the presence or absence of 10-fold trap DNA.

These results provide additional evidence that the observed annealing activity is intrinsic to Mcm2~7. Our results also indicate that ATP binding of each Mcm subunit differentially affects DNA helicase and DNA annealing activities. It was rather unexpected that ATP binding of Mcm4 and Mcm6 is required for DNA annealing activity, since the annealing activity itself does not require ATP (Figure 4). We speculate that ATP binding of Mcm subunits may modulate the conformation of the Mcm2~7 complex, thus affecting the DNA annealing activity. In support of this speculation, ATP-γ-S did not completely inhibit the annealing activity of Mcm2~7(2,5), while the same concentration of ATP-γ-S completely suppressed that of the wild-type Mcm2~7 (Figure 5D, lanes 12–14).

We noted that the level of displaced strand DNA was reduced at the highest concentration of Mcm2~7, where strong DNA binding is observed (Figure 5A and C, lane 4). This is probably due to the strong strand annealing activity of Mcm2~7 at this concentration (Figure 5D).

Effects of phosphorylation on strand annealing activity of Mcm2~7

It has been known that phosphorylation of Mcm complexes by Cdk or Chk2 kinase inhibits their helicase activity *in vitro* ([22–25]). Mcm2, Mcm4 and Mcm6 are the targets of Cdc7-mediated phosphorylation and Mcm4 and Mcm6 are known to be highly phosphorylated also by CDK kinases ([18–21]). Thus, it was tested whether the phosphorylation of Mcm2~7 affects the annealing activity. The Mcm2~7 was phosphorylated *in vitro* by Cdc7-ASK, Cdk2/Cyclin A, Cdc2/Cyclin B, and Cdk2/Cyclin E kinases. Mcm2, 4 and 6 subunits were phosphorylated by these kinases, as expected (Figure 6A). Phosphorylation of Mcm2~7 by these kinases significantly inhibited its DNA strand annealing activity (Figure 6B). These results suggest that the annealing activity of Mcm2~7 may be regulated by phosphorylation.

We also examined the effect of phosphorylation of Mcm2~7 on its DNA-binding activity. In gel shift assays with ^{32}P-labeled fork DNA and pull-down assays with a biotin-labeled fork DNA, the DNA-binding activity of Mcm2~7 was not significantly affected by phosphorylation with any kinases tested (Supplementary Figure SC and data not shown). Thus, the results suggest that reduced anneal-
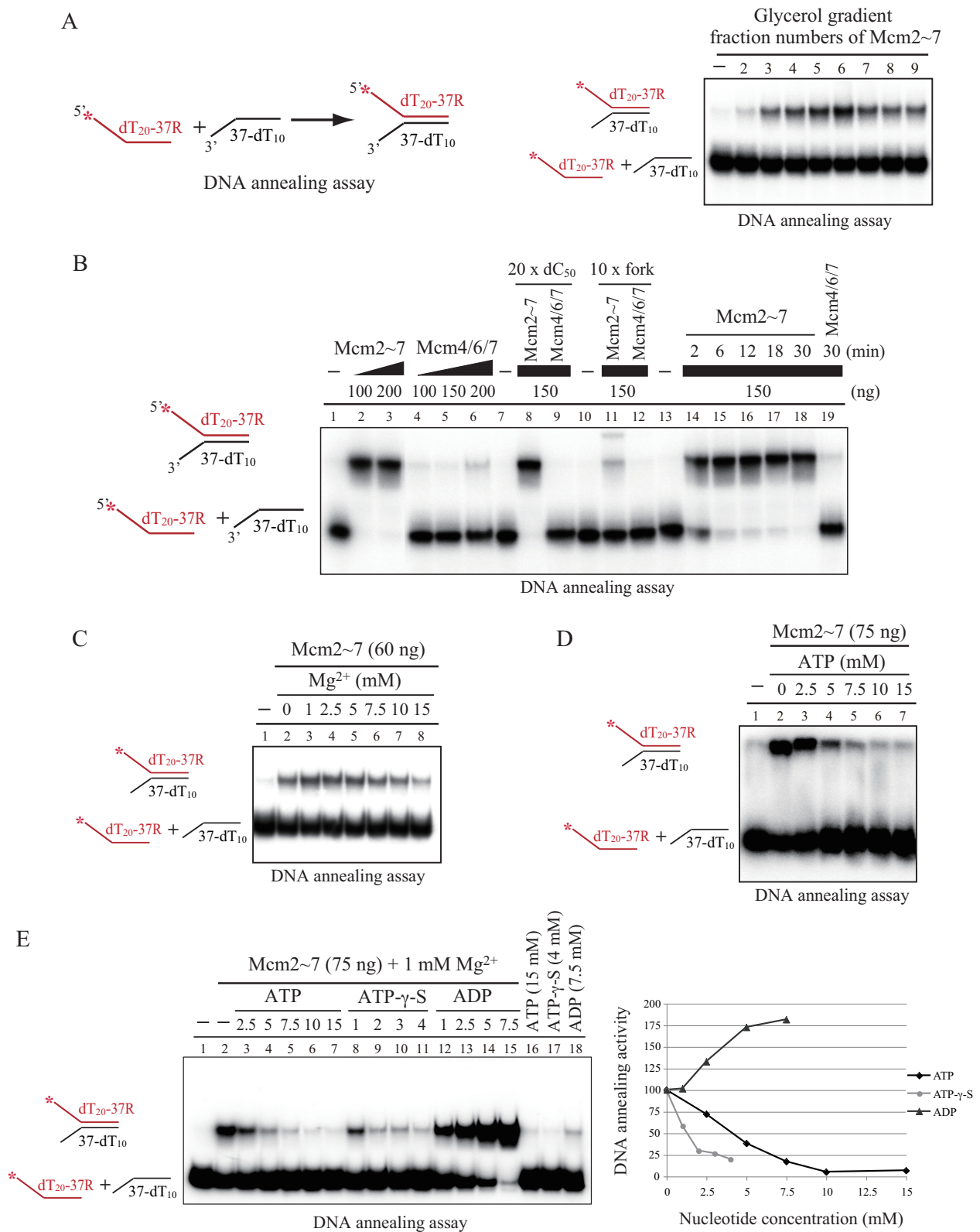
Figure 4. DNA strand annealing activity of Mcm2~7 and Mcm4/6/7. (**A**) Schematic representation of two partially complementary ssDNAs annealing to form a 37 bp forked DNA. DNA annealing assay of Mcm2~7 (glycerol gradient fractions used in Figure 3) was performed by mixing labeled dT$_{20}$-37R (10 fmol) and unlabeled 37-dT$_{10}$ (15 fmol) at 30°C for 30 min without Mg^{2+}-ATP. (**B**) The DNA annealing assays were conducted without magnesium and ATP. The strand annealing activity of Mcm4/6/7 and Mcm2~7 were examined in the presence of the ssDNA (dC$_{50}$) or fork DNA (dT$_{25}$-25/25-dT$_{25}$). The amount of annealed DNA was analyzed on 10% non-denaturing PAGE/1× TBE. The effects of various concentrations of Mg^{2+} (**C**) and ATP (**D**) on annealing activity of Mcm2~7 were examined. (**E**) Left, DNA annealing assays of Mcm2~7 were conducted in the presence of various concentrations of ATP, ATP-γ-S and ADP. Right, The levels of DNA annealing of Mcm2~7 were quantified and expressed as relative values with DNA annealing activity in the absence of nucleotide taken as 100. Square, ATP; circle, ATP-γ-S; triangle, ADP.
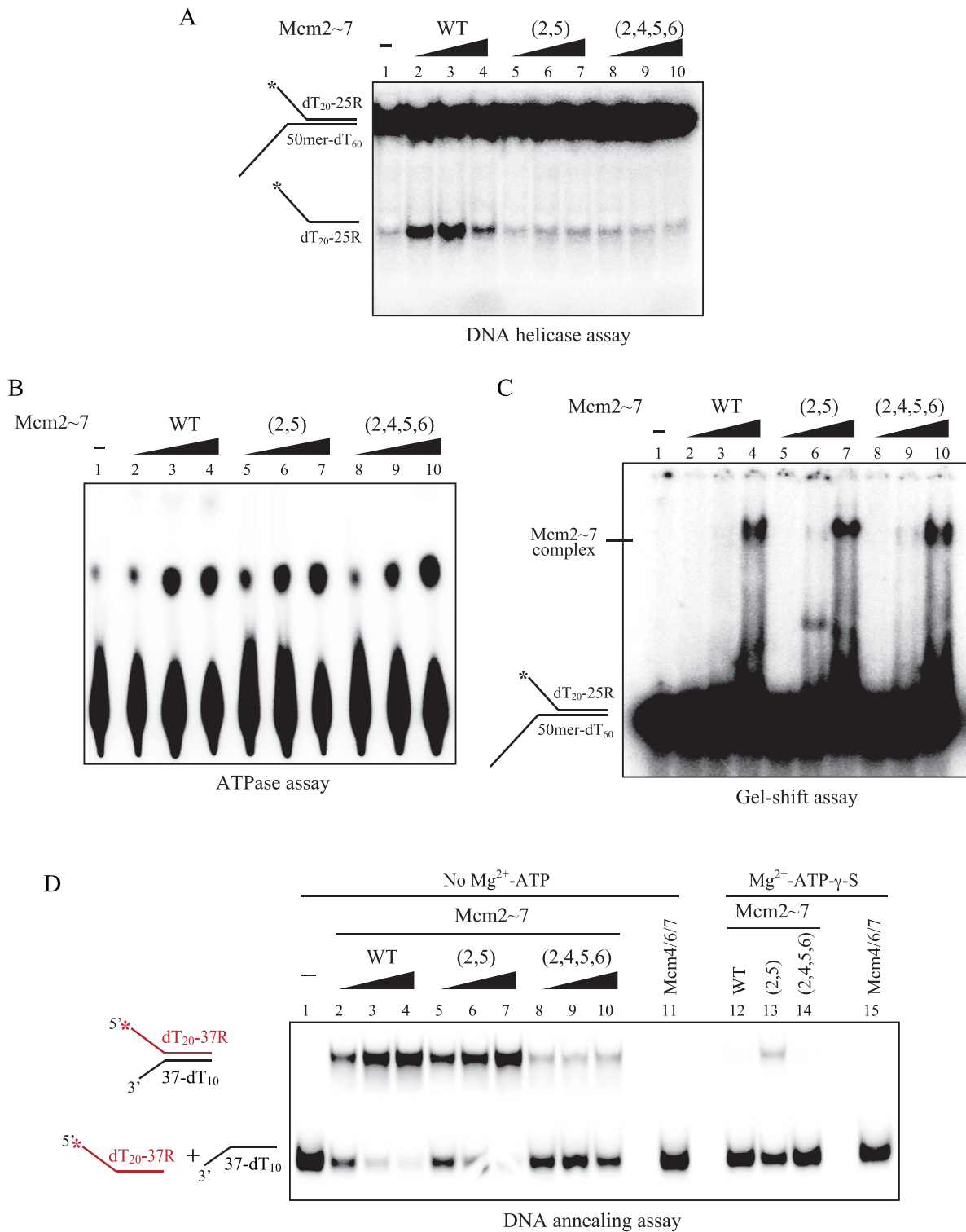
Figure 5. DNA helicase, DNA-binding, ATP hydrolysis and DNA annealing activities of the ATP binding mutant Mcm2∼7 complexes. DNA helicase (**A**) and DNA binding (**C**) activities were examined with wild-type and mutant Mcm2∼7 complexes (75, 150 and 300 ng). In DNA binding assays, a part of reaction (with 1 mM ATP-γ-S) was directly applied on a polyacrylamide gel containing 6 mM magnesium acetate and 5% glycerol in 0.5× TBE after incubation at 30°C for 30 min. In helicase assays, the remainder of reaction was further incubated at 37°C for 30 min after addition of 10 mM ATP before loading on 10% non-denaturing PAGE/1× TBE. The wild-type Mcm2∼7 showed trap DNA-dependent helicase activity, while two mutated Mcm2∼7 complexes were deficient in DNA unwinding activity. (**B**) ATPase activities were examined in the presence of a 87mer oligonucleotide (50 ng/μl). The amount of the wide-type and mutant Mcm2∼7 proteins used were; 120, 240 and 480 ng. (**D**) DNA annealing assays were carried out at 30°C for 30 min with the wide-type and mutant Mcm2∼7 proteins (37.5, 75 and 150 ng) using 10 fmol labeled dT_{20}-37R and 15 fmol unlabeled 37-dT_{10}. The reactions did not contain any magnesium acetate or ATP (lanes 1–11) or contained 5 mM Mg-acetate and 1 mM ATP-γ-S (lanes 12–15). After termination of the reactions, the samples were analyzed on 10% non-denaturing PAGE/1× TBE.
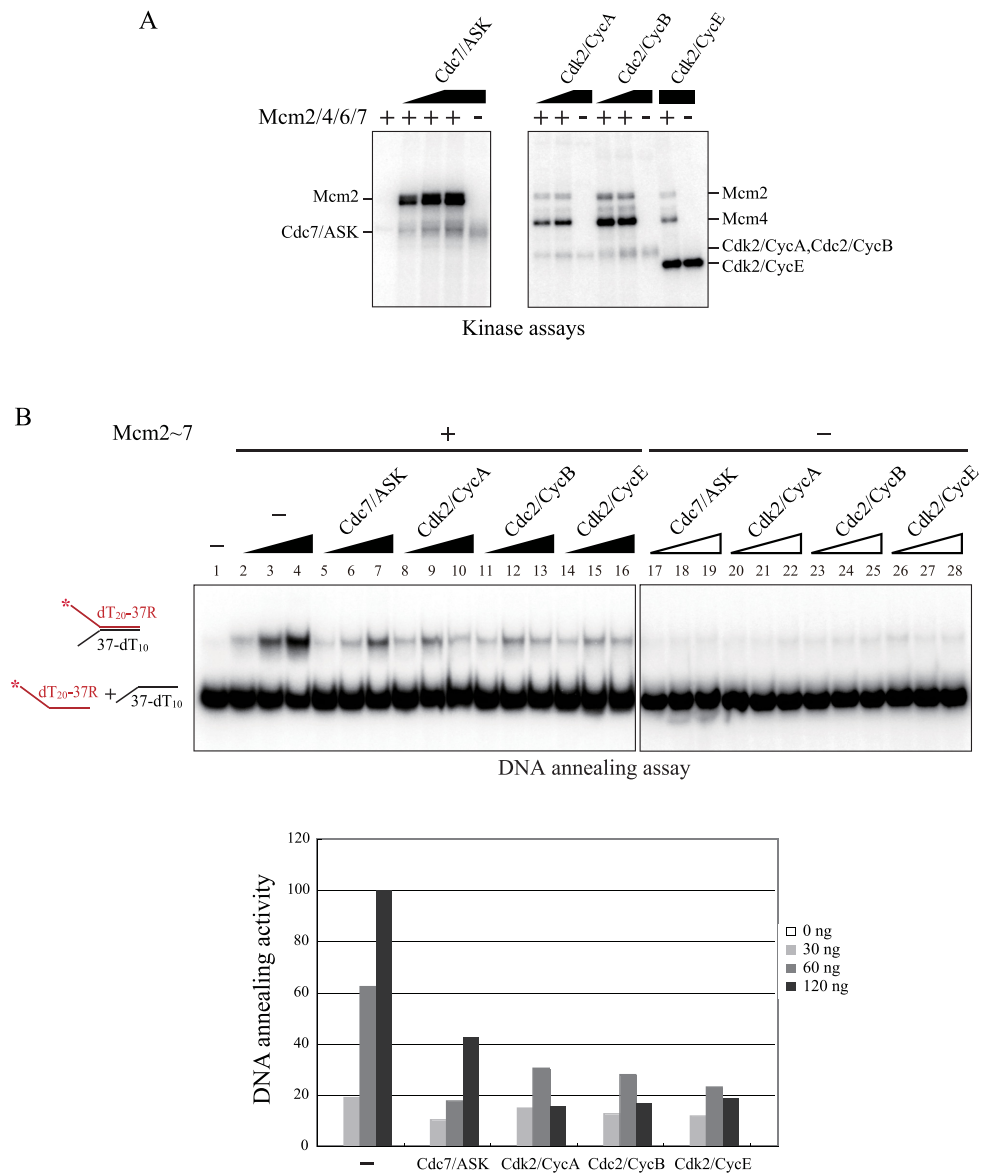
Figure 6. Effect of phosphorylation on annealing activity of Mcm2∼7. (**A**) *In vitro* phosphorylation assays of the Mcm2/4/6/7 complex were conducted with Cdc7/ASK, Cdk2/CycA, Cdc2/CycB or Cdk2/CycE in the presence of [γ-^{32}P]ATP. The reaction products were analyzed on 5–20% SDS-PAGE and dried for autoradiogram. (**B**) The Mcm2∼7 was phosphorylated by various kinases (Cdc7/ASK, Cdk2/Cyclin A, Cdc2/Cyclin B and Cdk2/Cyclin E) or non-treated *in vitro* and increasing amounts of the phosphorylation reactions (containing 30, 60 and 120 ng of Mcm2∼7) were used for the standard DNA annealing assay (lanes 2–16; shown by filled triangles). Identical phosphorylation reactions were conducted in the absence of Mcm2∼7, and increasing amounts of the reactions were also used for the DNA annealing assay (lanes 17–28; shown by open triangles). The products were analyzed on 10% non-denaturing PAGE/1× TBE. The levels of DNA annealing in the autoradiograms were quantified by Fuji image analyzer, expressed as relative values (the maximum level of strand annealing [lane 4] taken as 100) and were plotted.

ing activity of Mcm2∼7 by phosphorylation is not due to its effect on DNA binding activity.

Evaluation the DNA annealing activity of various Mcm complexes

In addition to the Mcm2∼7 heterohexameric complex, the Mcm proteins form other subcomplexes including Mcm4/6/7 hexamer (double-trimer), Mcm2/4/6/7 tetramer and Mcm3/5 dimer. In order to dissect the potential roles of each subunit of Mcm2∼7 in its DNA anneal-

ing activity, we purified these Mcm subcomplexes as well as Mcm2 single polypeptide and examined their ability to anneal ssDNA. The Mcm3/5 complex, highly purified on glycerol gradient centrifugation, exhibited strong annealing activity (Figure 7A and B), although the purified Mcm3/5 did not show any significant DNA binding activity in pull down or gel-shift assays using a Y-fork substrate (data not shown). DNA strand annealing activity of Mcm3/5 was inhibited by increasing concentrations of ATP-γ-S and ATP, whereas it was stimulated by ADP (Figure 7C), as found with Mcm2∼7. Strong strand annealing activity was de-
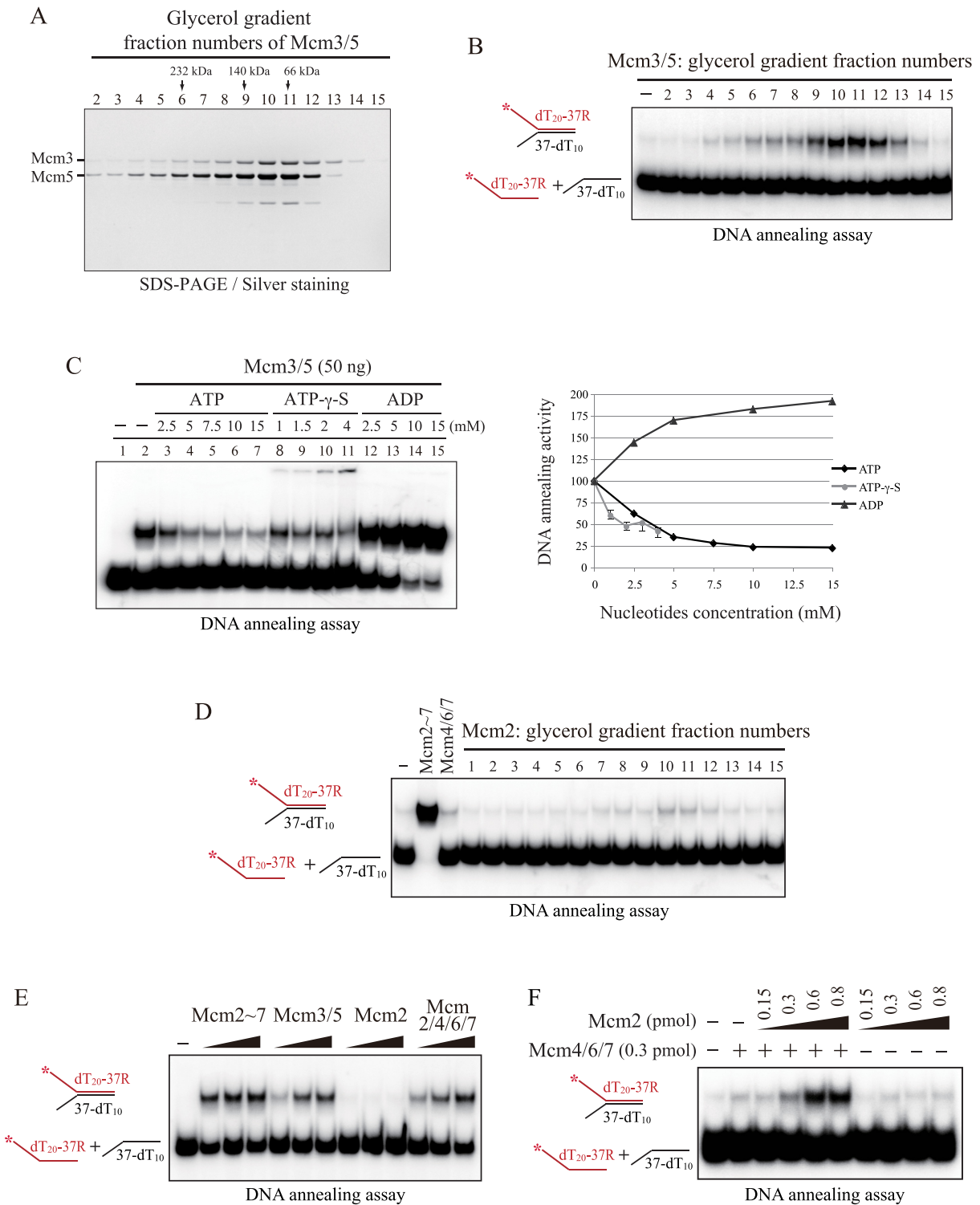
Figure 7. DNA annealing activity of various Mcm complexes. (**A**) Mcm3/5 complex were purified with several steps and were finally fractionated on 15–35% glycerol gradient at 36,000 rpm for 17 h. Each fraction was electrophoresed in SDS-PAGE and stained with silver. The amount of Mcm5 appears to be larger than that of Mcm3 in silver staining, but we confirmed that each subunit is present in an equal stoichiometry after staining with Coomassie Brilliant Blue (data not shown). (**B**) The annealing activity of Mcm3/5 was examined under the standard assay condition. (**C**) Left, DNA annealing assays of Mcm3/5 were conducted in the presence of various concentrations of ATP, ATP-γ-S and ADP. Right, The levels of DNA annealing of Mcm3/5 were quantified and expressed as relative values with DNA annealing activity in the absence of nucleotide taken as 100. Square, ATP; circle, ATP-γ-S; triangle, ADP. (**D**) Mcm2 polypeptide was purified on glycerol gradient centrifugation (see Supplementary Figure SE and SF) and its annealing activity was examined under the standard assay condition. (**E**) Comparison of the specific DNA annealing activity of various Mcm complexes. The equal amounts (0.1, 0.2 and 0.4 pmol) of Mcm2~7, Mcm3/5, Mcm2 alone or Mcm2/4/6/7 were used in annealing assays. (**F**) Effect of Mcm2 on the annealing activity of the mcm4/6/7 complex. Increasing amounts of Mcm2 protein were added to a constant amount of the Mcm4/6/7 complex in a standard annealing assay.

tected also in the purified Mcm2/4/6/7 tetramer (Supplementary Figure SD). In contrast, only a very low annealing activity was observed in the purified Mcm2 polypeptide (Figure 7D; Supplementary Figure SE and SF). We determined the specific activities of DNA annealing of various Mcm complexes; the relative specific activities of Mcm3/5, Mcm2/4/6/7 and Mcm2 were 0.75, 0.83 and 0.064, respectively, with that of Mcm2∼7 complex taken as 1 (Figure 7E).

We previously reported that the Mcm2 and Mcm3/5 inhibit the helicase activity of Mcm4/6/7 by converting its double trimeric structure into a heterotetramer and heteropentamer, respectively (6,7). We then examined whether addition of Mcm2 to Mcm4/6/7 would lead to increased DNA annealing activity. Addition of stoichiometric amount of purified Mcm2 polypeptide to the purified Mcm4/6/7 complex resulted in significant strand annealing activity (Figure 7F), consistent with the generation of the Mcm2/4/6/7 complex (6). This result also indicates that DNA annealing activity of Mcm may not reside in a single Mcm subunit but may be mediated by the specific Mcm complexes.

DISCUSSION

In this study, we report that the mouse Mcm2∼7 complex possesses an intrinsic DNA annealing activity. In the presence of a trap DNA that is complementary to displaced unlabeled strand of DNA, which prevents reannealing of the displaced labeled DNA, the DNA unwinding activity of Mcm2∼7 could be detected. Our result strongly suggests that the mammalian Mcm2∼7 heterohexameric complex has intrinsic DNA helicase activity, but it has not been detected in a standard reaction condition, likely due to its strong DNA annealing activity.

Possible mechanisms of Mcm2∼7 DNA annealing activity

The inhibition of the annealing activity by a fork DNA but not by ssDNA (Figure 4B) suggests the involvement of the fork binding activity of Mcm2∼7 in its ability to anneal DNA. However, Mcm2∼7(2,4,5,6) is deficient in annealing while it is fully active in fork DNA binding (Figure 5C and D). Phosphorylation reduces DNA strand annealing activity but does not affect fork binding. Thus, fork DNA binding alone does not explain the strand annealing activity of Mcm2∼7.

The DNA strand annealing activity of Mcm2∼7 does not require Mg^{2+} or ATP, and is adversely inhibited by the presence of high concentration of Mg^{2+} and ATP. The estimated intracellular ATP concentration in eukaryotic cells is in the low millimolar range, and the cellular ADP concentration is five-fold lower than that of ATP (53–55). Thus, cellular endogenous ATP may by somewhat inhibitory for DNA annealing activity of Mcm2∼7, whereas cellular ADP level would be not be high enough to stimulate it. Thus, cellular environment in general may be more favorable for DNA unwinding.

We show that mutations of ATP binding motifs in Mcm subunits differentially affected DNA annealing activity. Mcm2∼7(2,5) showed the wild-type level of annealing activity while Mcm2∼7(2,4,5,6) was defective in DNA anneal-

ing. Both Mcm2∼7(2,5) and Mcm2∼7(2,4,5,6) were deficient in DNA unwinding, thus suggesting that the annealing and unwinding activities of Mcm2∼7 exhibit distinct structural requirement. Since high concentration of ATP inhibits the annealing activity, it is likely that ATP induces conformational change of Mcm2∼7, in such a way that its annealing activity is suppressed. This is also supported by the fact that DNA annealing activity of Mcm2∼7(2,5) mutant is less affected by ATP-γ-S (Figure 5D), suggesting that ATP binding of Mcm2 and Mcm5 may be involved in induction of annealing-deficient conformation. Similar inhibition of strand annealing activity by ATP was previously reported for several helicases that also possess annealing activity, including the RECQ5β and RECQ1 (31,37,44). Thus, annealing activity of Mcm2∼7 depends on specific conformation of the complex as well as on its fork binding activity. Indeed, biochemical and structural studies have shown that the nucleotides and DNA induced conformational change in the Mcm2∼7 helicase (4,56–58).

We show that not only Mcm2∼7 but also Mcm2/4/6/7 and Mcm3/5 subcomplexes are associated with strong DNA annealing activity, whereas Mcm2 or Mcm4/6/7 showed very little DNA annealing activity. Addition of Mcm2 converted the annealing-deficient Mcm4/6/7 into the annealing-proficient Mcm2/4/6/7 complex. Our results strongly suggest that DNA annealing activity of Mcm2∼7 is mediated by specific Mcm assemblies but not by a single Mcm subunit.

Regulation of DNA annealing and helicase activities of Mcm2∼7 complex

Phosphorylation of Mcm2∼7 by Cdc7 or by CDK inhibited its DNA annealing activity (Figure 6B), suggesting a possibility that the phosphorylation events may directly contribute to the activation of the Mcm replicative helicase through inactivation of its DNA annealing activity. Phosphorylation also has been shown to inhibit DNA helicase activity *in vitro* (22–25). Thus, effects of phosphorylation on DNA annealing and helicase activities are complex. Phosphorylation of specific sites by a specific kinase may differentially affect annealing or helicase activity. More detailed analyses of effect of phosphorylation on Mcm2∼7 activities need to be conducted. After the pre-RC is activated by Cdc7 and CDK, Cdc45 and GINS associate with pre-RC, resulting in the formation of the Cdc45-Mcm2∼7-GINS (CMG) helicase complex (3,4,12,15,59). Thus, it is also possible that strong DNA annealing activity of Mcm2∼7 is suppressed by its association with Cdc45 and GINS. These possibilities need to be further examined by using the purified mammalian CMG complex.

Potential roles of DNA strand annealing activity of Mcm in stabilization of a stalled replication fork

In the presence of DNA lesions, replication forks are stalled, and could collapse in the presence of unrepaired ssDNA. Annealing helicases might stabilize stalled replication forks through annealing the exposed ssDNAs that may have been generated by uncoupling of the helicase and replication machinery or through promoting fork reversal to facilitate the

repair of the lesion. A recent work reported that Mcm could be recruited to the sites of DNA damage, suggesting that Mcm proteins may be involved in DNA repair as well (28). Mcm is phosphorylated by checkpoint kinases in response to replication stress (21,25,60). The effect of this phosphorylation on DNA annealing activity of Mcm needs to be examined. CMG helicase has been reported to disassemble at collapsed replication forks (61). This might activate the strand annealing activity of Mcm2∼7 for fork stabilization. The biological roles of DNA annealing activity of Mcm need to be examined by generating a mutant Mcm that is specifically defective in DNA strand annealing but is proficient in other functions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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