ATP Binding and Hydrolysis by Mcm2 Regulate DNA Binding by Mcm Complexes

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Abstract

The essential minichromosome maintenance (Mcm) proteins Mcm2 through Mcm7 likely comprise the replicative helicase in eukaryotes. In addition to Mcm2–7, other subcomplexes, including one comprising Mcm4, Mcm6, and Mcm7, unwind DNA. Using Mcm4/6/7 as a tool, we reveal a role for nucleotide binding by *Saccharomyces cerevisiae* Mcm2 in modulating DNA binding by Mcm complexes. Previous studies have shown that Mcm2 inhibits DNA unwinding by Mcm4/6/7. Here, we show that interaction of Mcm2 and Mcm4/6/7 is not sufficient for inhibition; rather, Mcm2 requires nucleotides for its regulatory role. An Mcm2 mutant that is defective for ATP hydrolysis (K549A), as well as ATP analogues, was used to show that ADP binding by Mcm2 is required to inhibit DNA binding and unwinding by Mcm4/6/7. This Mcm2-mediated regulation of Mcm4/6/7 is independent of Mcm3/5. Furthermore, the importance of ATP hydrolysis by Mcm2 to the regulation of the native complex was apparent from the altered DNA binding properties of Mcm2^{KA}–7. Moreover, together with the finding that Mcm2_{K549A} does not support yeast viability, these results indicate that the nucleotide-bound state of Mcm2 is critical in regulating the activities of Mcm4/6/7 and Mcm2–7 complexes.

Keywords

DNA replication; helicase; ATPase; DNA binding; Mcm

Introduction

Control of DNA replication is essential for the growth and differentiation of cells and is often disrupted in diseased states. A necessary step in DNA replication is the separation of duplex DNA strands by a replicative helicase. In eukaryotic cells, the replicative helicase is likely a complex comprising six related proteins termed minichromosome maintenance (Mcm) proteins, numbered 2 through 7 (Mcm2–7).¹ Consistent with their proposed role as components of the replicative helicase, complexes from *Xenopus* eggs and *Drosophila* embryos that include Mcm2–7 are able to unwind DNA.^{2,3} In addition, each protein is

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Supplementary Data

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required for both the initiation and the elongation stages of DNA replication, and each encodes ATP sites that are essential for function (reviewed by Forsburg¹).

All six Mcm2–7 proteins are required for DNA replication in *Saccharomyces cerevisiae*;⁴ however, DNA unwinding by Mcm2–7 assembled onto origins does not occur until after an activation step. Activation is thought to require posttranslational modification of Mcm2–7 by Dbf4-dependent kinase (DDK) and interaction of Mcm2–7 with the replication fork proteins GINS (*go*, *ishi*, *ni*, *san*, a complex comprising Sld5, Psf1, Psf2, and Psf3) and cell division control (Cdc) 45.^{2,3,5–7} This may explain why DNA unwinding by Mcm2–7 has not been detected *in vitro*, except in complexes that contain Cdc45 and GINS.^{2,3} However, Bochman and Schwacha recently reported that, under certain ionic conditions, DNA unwinding by purified Mcm2–7 complexes can be achieved *in vitro*.⁸ Interestingly, a subcomplex comprising Mcm4, Mcm6, and Mcm7, initially purified from HeLa cells, unwinds DNA *in vitro* under a variety of conditions.^{9–11} Furthermore, DNA unwinding by this complex is inhibited by Mcm2 and Mcm3/5.^{9–13} Explanations of these diverse observations include the idea that while all six subunits of Mcm2–7 may be required for DNA unwinding, not all participate *directly* in the catalysis of DNA unwinding and that, within the context of a replication origin, activation of Mcm2–7 by DDK, GINS, and Cdc45 involves the relief of inhibition by Mcm2 and Mcm3/5.

The ability of Mcm2 to inhibit Mcm4/6/7 is the starting point of the studies presented here. Current models suggest that Mcm2 modulates the helicase activity of Mcm4/6/7 by disrupting the oligomeric structure of the complex. The functional form of Mcm4/6/7 is likely a hexameric ring comprising two Mcm4/6/7 trimers that bind to DNA by encircling it within a central channel.^{9–11,14–16} This mode of DNA interaction is likely important for DNA unwinding.^{10,17–19} Based on the observation that Mcm2 interacts with Mcm4/6/7 in the absence of ATP and disrupts the hexameric structure to form tetramers containing one of each protein,^{9,11,20} current models propose that modulation of Mcm4/6/7 activity by Mcm2 occurs by a “passive” process and is simply a consequence of the interaction between Mcm2 and Mcm4/6/7.

Here, we examine the mechanism of the Mcm2 modulation of Mcm4/6/7 activities and relate these findings to the Mcm2–7 complex from *S. cerevisiae*. Specifically, we tested the hypothesis that Mcm2 inhibits Mcm4/6/7 activity via a passive mechanism using an Mcm2 mutant (Mcm2_{K549A}) that was severely compromised in its ability to hydrolyze ATP with the expectation that the mutant would inhibit Mcm4/6/7 activity. Surprisingly, we found that Mcm2_{K549A} was unable to inhibit DNA unwinding by Mcm4/6/7. This same mutant interacted with Mcm4/6/7, suggesting that the association of Mcm2 with Mcm4/6/7 is not sufficient for Mcm2 to exert its effects. Rather, based on observations with ATP analogues and Mcm2_{K549A}, we suggest that the ADP-bound state of Mcm2 is required for inhibition of DNA binding and, thus, DNA unwinding by Mcm4/6/7. The relevance of this result to the intact complex was apparent from the finding that the Mcm2–7 complex containing Mcm2_{K549A} in place of wild-type Mcm2 possesses a higher affinity for single-stranded DNA (ssDNA) than wild-type Mcm2–7. We suggest that ATP hydrolysis at the Mcm2 site modulates Mcm2–7 DNA binding by a mechanism in which the ATP binding status of Mcm2 is communicated to other Mcm subunits.

Results

Mutation of the Mcm2/6 interface disrupts ATP hydrolysis

The Mcm2–7 complex contains six ATP sites encoded by each of the subunits that are important for the function of this complex.¹ To determine the effects of ATP hydrolysis by Mcm2 on its potential function as a regulator, we mutated the invariant lysine residue (K549) of the Mcm2 P-loop, a motif found in many nucleotide-binding proteins,^{21,22} to alanine. Previous studies have indicated that the *mcm2*_{K549A} allele does not support viability.^{23,24}

ATP hydrolysis by Mcm2 is dependent on Mcm6.^{23,25} The ATP site of Mcm2/6 is located at the interface of the two proteins, with a conserved arginine residue from Mcm6 contributing to the hydrolysis of ATP bound to Mcm2²⁵ (Fig. 1a). Mcm2_{K549A} and wild-type Mcm2 were purified identically from *Escherichia coli* expression strains, and ATP hydrolysis was assayed. Neither Mcm2 nor Mcm6 alone hydrolyzed ATP at rates that were significantly above background levels seen in the absence of protein ($0.5 \pm 0.1 \text{ min}^{-1}$; Fig. 1b). However, when Mcm2 and Mcm6 were mixed, ATP hydrolysis, roughly 10-fold above levels with individual proteins, was detected ($4.9 \pm 0.2 \text{ min}^{-1}$; Fig. 1b and c). These results confirmed that ATPase activity requires both Mcm2 and Mcm6 and verified that there were no contaminating ATPases in individual preparations. Substitution of Mcm2_{K549A} for wild-type Mcm2 had a severe effect on ATP hydrolysis, with a rate around background levels ($0.3 \pm 0.1 \text{ min}^{-1}$). Also consistent with the proposed arrangement in Fig. 1a, mutation of the conserved catalytic arginine in Mcm6 to an alanine (Mcm6_{R708A}) had the same effect on ATP hydrolysis as the Mcm2_{K549A} mutant. Mcm6_{R708A}, in combination with Mcm2, hydrolyzed ATP with a rate around background levels ($0.3 \pm 0.1 \text{ min}^{-1}$; Fig. 1b and c). Our Mcm2/6 ATPase analyses indicate that both the conserved lysine 549 of Mcm2 and arginine 708 of Mcm6 are required for ATP hydrolysis by Mcm2/6. The reduced hydrolysis observed with each of the mutants may be due to a direct effect on catalysis and/or reduced ATP binding. We detected interactions between Mcm2 and Mcm6 with each of the mutant proteins, indicating that the lack of ATPase activity is not due to a lack of protein–protein interactions (Supplementary Information).

Mcm2_{K549A} interacts with Mcm4/6/7

Previous studies in *Schizosaccharomyces pombe* and mammalian systems have demonstrated that Mcm2 forms a complex with Mcm4/6/7 in the absence of ATP.^{9,11,20} To determine whether Mcm2_{K549A} is altered in its interaction with Mcm4/6/7, we examined Mcm2 interaction with Mcm4/6/7 by taking advantage of a histidine tag on the N-terminus of Mcm6²⁵ that allows Mcm4/6/7 to bind nickel (Ni) affinity resin (Fig. 2a, lane 3). In contrast, Mcm2 only binds Ni resin if it is bound to Mcm4/6/7 (Fig. 2a, lanes 6 and 9; Fig. 2b, lanes 3 and 5). Equimolar ratios of Mcm2 (monomer) and Mcm4/6/7 (hexamer) with 5 mM ATP were used in these assays. When the binding experiment was repeated with Mcm2_{K549A}, a comparable level of interaction between Mcm2_{K549A} and Mcm4/6/7 was detected (Fig. 2a, lane 12; Fig. 2b, lane 6). The ability of Mcm2_{K549A} to interact with Mcm4/6/7 was confirmed by coelution of Mcm2_{K549A} with Mcm4/6/7 through gel-filtration

columns in the presence and in the absence of ATP (Supplementary Information and data not shown).

Mcm2_{K549A} modulates ATP hydrolysis by Mcm4/6/7

A study with *S. pombe* proteins has shown that Mcm2 almost completely inhibits ATP hydrolysis by Mcm4/6/7.¹¹ When we tested the effects of Mcm2 on ATP hydrolysis by *S. cerevisiae* Mcm4/6/7, a decrease to 68% of activity in the absence of Mcm2 was detected ($40.6 \pm 6.0 \text{ min}^{-1}$ without Mcm2 and $27.8 \pm 5.5 \text{ min}^{-1}$ with Mcm2; Fig. 3). Increasing the molar ratio of Mcm2 to Mcm4/6/7 did not increase inhibition (data not shown). To examine whether ATP hydrolysis contributed by Mcm2 masks any reduction in Mcm4/6/7 activity, we turned to the mutant version of Mcm2. In the presence of Mcm2_{K549A}, ATP hydrolysis by Mcm4/6/7 was reduced to $11.5 \pm 5.1 \text{ min}^{-1}$ (Fig. 3). These results suggest that Mcm2 modulates ATP hydrolysis by Mcm4/6/7, even in the absence of ATP hydrolysis by Mcm2. Furthermore, the inhibition of Mcm4/6/7 by Mcm2_{K549A} confirms the ability of the mutant to interact with Mcm4/6/7.

Inhibition of DNA unwinding by Mcm4/6/7 requires ATP binding and/or hydrolysis by Mcm2

Mcm2 inhibits DNA unwinding by purified Mcm4/6/7 complexes from a variety of eukaryotic systems.^{9–13} Current models suggest a passive mechanism of inhibition, which simply requires the interaction of Mcm2 and Mcm4/6/7.^{9,11} In contrast, active regulation of Mcm4/6/7 DNA unwinding would require the essential ATP site of Mcm2. We examined the regulation of DNA unwinding by Mcm4/6/7 using oligonucleotide substrates established previously.¹⁰ DNA unwinding is detected as the appearance of ssDNA product accompanied by the loss of double-stranded DNA (dsDNA) fork on native polyacrylamide gels (Fig. 4a). To examine whether mutation of the essential Mcm2 ATP site affected its ability to modulate Mcm4/6/7 helicase activity, we titrated wild-type Mcm2 (lanes 4–6) or Mcm2_{K549A} (lanes 7–9) into a constant concentration of Mcm4/6/7. The percent DNA unwound was calculated as the proportion of total DNA that migrates as ssDNA on the gel. DNA unwinding was inhibited at all concentrations of Mcm2 assayed, reaching a maximum at equimolar ratios of Mcm2 monomer to Mcm4/6/7 hexamer (Fig. 4b, circles). In contrast, Mcm2_{K549A} was unable to inhibit DNA unwinding by Mcm4/6/7 (Fig. 4b, squares). Neither wild type nor Mcm2_{K549A} contained contaminating helicase or nuclease activities that would interfere with these results (Fig. 4a, lanes 10 and 11). We concluded that ATP binding or hydrolysis by Mcm2 is required for the regulation of DNA unwinding by Mcm4/6/7. Furthermore, these results indicate that Mcm2_{K549A} is a valuable tool for evaluating models of the mechanism by which Mcm2 inhibits helicase activity.

DNA binding by Mcm4/6/7

Mcm2 may inhibit the helicase activity of Mcm4/6/7 by interfering with DNA binding by Mcm4/6/7,^{12,15,26} a necessary prelude to DNA unwinding. Before examining whether Mcm2_{K549A} inhibits ssDNA binding by Mcm4/6/7, we first established an ssDNA-binding assay. We used a gel-filtration-based approach to examine the binding of Mcm4/6/7 to circular DNA similar to assays previously used to examine DNA binding by the *E. coli* replicative helicase DnaB.²⁷ One advantage of this assay is that it utilizes circular DNA; thus, helicases cannot translocate off the ends, as may occur on linear DNA. We used a gel-

filtration column in which DNA and any bound protein elute at a relatively small volume, while free protein elutes later. We radiolabeled Mcm4/6/7 by engineering a protein kinase A (PKA) target site on the N-terminus of Mcm4. The protein Mcm4^{PK} was purified, reconstituted into a complex with Mcm6 and Mcm7, and then radiolabeled to high specific activity using [γ ³²P]ATP and PKA. When Mcm4^{PK}/6/7 was applied to the gel-filtration column in the absence of ssDNA, the majority of the protein eluted in fractions 19–29 (Fig. 5a, triangles). Mixing Mcm4^{PK}/6/7 and M13mp18 ssDNA, followed by gel filtration, resulted in most of Mcm4^{PK}/6/7 coeluting with DNA in earlier fractions (10–15), indicating that DNA binding by Mcm4^{PK}/6/7 had occurred (Fig. 5a, squares). The elution profile of ssDNA through the gel-filtration column was confirmed by agarose gel electrophoresis in a separate experiment, and the presence of Mcm6 and Mcm7 subunits in ssDNA-containing fractions was verified by Western blot analysis of sample experiments (data not shown). We also examined whether Mcm4^{PK}/6/7 binds to circular dsDNA, using a 7.3-kb double-stranded plasmid in place of M13mp18, and observed that Mcm4^{PK}/6/7 does not bind dsDNA to the same extent as ssDNA (Fig. 5a, circles), consistent with a previous study.¹⁴

The experiments described above were performed in the presence of 5 mM ATP. When ATP was omitted from DNA binding assays, very little binding of Mcm4^{PK}/6/7 to ssDNA was detected (Fig. 5b, circles). We also examined the effects of ADP and the slowly hydrolyzed analogue adenosine 5'-O-(3-thio)triphosphate (ATP γ S) on DNA binding by Mcm4^{PK}/6/7. When ADP was provided to Mcm4^{PK}/6/7 in place of ATP, binding was observed, but was slightly reduced to 80 $\pm$ 10% of Mcm4^{PK}/6/7 bound in the presence of ATP (Fig. 5c, circles). In contrast, there was no decrease in ssDNA binding by Mcm4/6/7 when ATP γ S was provided in place of ATP (Fig. 5d, circles). Very little Mcm4^{PK}/6/7 was observed in early fractions in the absence of ssDNA with any of the nucleotides (Fig. 5a and data not shown). In summary, we have shown that Mcm4/6/7 binds to ssDNA circles in a nucleotide-dependent manner and that DNA binding with ATP and ATP γ S is slightly greater than that with ADP.

Mcm2 requires ATP hydrolysis to inhibit ssDNA binding by Mcm4/6/7

In the presence of ATP, Mcm2 reduced the ssDNA binding of Mcm4^{PK}/6/7 to 65 $\pm$ 8%, the level seen when Mcm2 is absent (Fig. 6a, circles). We also examined the effects of ADP and ATP γ S on the ability of Mcm2 to inhibit ssDNA binding by Mcm4/6/7. In the presence of the poorly hydrolyzed ATP analogue ATP γ S, Mcm2 did not inhibit DNA binding of Mcm4^{PK}/6/7 (Fig. 6b, circles). This suggests that ATP hydrolysis by Mcm2 is required for inhibition of Mcm4/6/7. ATP γ S is likely bound by Mcm2 since it inhibits ATP hydrolysis by Mcm2/6 (data not shown). In contrast, ADP supported Mcm2-mediated inhibition to the same extent as was observed with ATP (reduction to 65 $\pm$ 9% of the amount of Mcm4^{PK}/6/7 bound to ssDNA when Mcm2 is absent; Fig. 6c, circles). Thus, it appears that the ADP-bound state of Mcm2 is required to inhibit Mcm4/6/7 binding to ssDNA. When ATP γ S is provided, inhibition may not occur, since it cannot be hydrolyzed to ADP. An alternative explanation is that the different nucleotides exert their effects on Mcm4/6/7 by changing its susceptibility to Mcm2. To differentiate between these scenarios, we returned to the Mcm2 mutant and examined its ability to regulate ssDNA binding by Mcm4/6/7. Mcm2_{K549A} did not inhibit DNA binding of Mcm4^{PK}/6/7 in the presence of ATP (Fig. 6a, triangles). This

observation suggests that ATP hydrolysis by Mcm2 is required for inhibition. However, the same result would occur if Mcm2_{K549A} were defective in nucleotide binding. To test this possibility, we examined the ability of Mcm2_{K549A} to inhibit Mcm4^{PK}/6/7 in the presence of ADP. Recall that ADP supports inhibition by wild-type Mcm2. Mcm2_{K549A} inhibited Mcm4^{PK}/6/7 binding to ssDNA to 69±6%, the level seen in the absence of Mcm2 (Fig. 6c, triangles), which is about the same extent as that seen with wild-type Mcm2 (Fig. 6c, compare circles to triangles). Thus, we conclude that ATP hydrolysis by Mcm2 is required for it to inhibit DNA binding and unwinding by Mcm4/6/7.

We tested whether Mcm2 associates with DNA-bound Mcm4/6/7 by performing a quantitative Western blot analysis on fractions from DNA binding experiments with Mcm4/6/7 and mutant or wild-type Mcm2. Mcm2 coeluted with M13 and Mcm4^{PK}/6/7 in amounts proportional to the amount of Mcm4^{PK}/6/7 (Fig. 6d). These results emphasize that the interaction of Mcm2 with Mcm4/6/7 is not sufficient for the inhibition of DNA binding by Mcm4/6/7.

Mcm2_{K549A} alters DNA binding of the Mcm2–7 complex

DNA unwinding *in vivo* requires all six Mcm2–7 subunits; therefore, we tested whether Mcm2 ADP-mediated regulation of DNA binding applied to this complex. We reconstituted both a wild-type Mcm2–7 complex and a complex containing the Mcm2_{K549A} mutant (Mcm2_{K549A}–7) and compared their activities. Given that Mcm2 inhibits Mcm4/6/7 DNA binding although Mcm2_{K549A} does not (which can also be stated as Mcm2_{K549A}/4/6/7 having a higher affinity for DNA than Mcm2/4/6/7), we anticipated that Mcm2_{K549A}–7 would have enhanced DNA binding compared to wild-type Mcm2–7. The Mcm2–7 complexes were formed with a PKA target sequence on the Mcm3 subunit. The PKA tag did not affect the activity of the complex and allowed labeling to high specific activity with ³²P (data not shown). We assayed for DNA binding by Mcm2–7^{3PK} and Mcm2_{K549A}–7^{3PK} using gel filtration. Both Mcm2–7^{3PK} and Mcm2_{K549A}–7^{3PK} require nucleotides to bind ssDNA and do not bind dsDNA (Fig. 7a–e; data not shown). We next tested the relative affinities of Mcm2–7^{3PK} and Mcm2_{K549A}–7^{3PK} for ssDNA binding in the presence of ATP by decreasing the amount of DNA in the assay with a constant concentration of Mcm2–7. Decreasing the ssDNA concentration from 100 fmol to 50 fmol resulted in an 8-fold reduction in DNA binding by Mcm2–7^{3PK} (Fig. 7c–f). In contrast, Mcm2_{K549A}–7^{3PK} bound DNA robustly at all DNA concentrations tested, even at the level of DNA that Mcm2–7^{3PK} bound very poorly (50 fmol; Fig. 7e and f). Consistent with our DNA binding studies of Mcm4/6/7, removal of the ATP site in Mcm2 enhances DNA binding of Mcm complexes.

An Mcm2 mutant with minimal ATP hydrolysis regulates Mcm4/6/7 and supports viability

The observation that ATP hydrolysis by Mcm2 regulates DNA binding by Mcm2–7 raises the possibility that ATP hydrolysis by Mcm2 has a regulatory role—and not a direct role—in the catalysis of DNA unwinding by Mcm2–7. Support for this idea comes from the observation that Mcm2_{K549A} does not hydrolyze ATP, yet is able to support DNA unwinding by Mcm2–7, albeit at a level lower than that of wild-type Mcm2–7.⁸ If ATP hydrolysis by Mcm2 is regulatory, then one might expect that an intermediate level of hydrolysis would be sufficient to support its activity. To explore this idea, we made a more conservative

substitution at the P-loop lysine by substituting lysine 549 with an arginine (Mcm2_{K549R}). This mutation resulted in a less drastic effect on ATP hydrolysis than Mcm2_{K549A} when mixed with Mcm6, reducing ATP hydrolysis to 18% of the activity with wild-type Mcm2 ($0.9 \pm 0.0 \text{ min}^{-1}$; Fig. 8a). Next, we examined whether the mutations in Mcm2 affect the viability of cells on rich media. We compared the growth of *S. cerevisiae* when plasmids bearing *MCM2*, *mcm2*_{K549R}, or *mcm2*_{K549A} were present as the sole copy of *MCM2* after shuffling out a wild-type copy of *MCM2* present on a *URA3* plasmid. Strains containing wild-type *MCM2* or *mcm2*_{K549R} were able to grow on plates containing 5-fluoroorotic acid (5-FOA), which selects for loss of the *URA3*-containing plasmid bearing *MCM2* (Fig. 8b). As observed previously,^{23,24} *mcm2*_{K549A} was unable to support growth. The lower level of ATP hydrolysis observed with Mcm2_{K549R} was sufficient for viability at all temperatures tested (Fig. 8b) and is consistent with the ability of this mutant to modulate Mcm4/6/7 DNA unwinding to the same extent as that of wild-type Mcm2 (Fig. 8c). These data suggest that ATP hydrolysis by Mcm2 is essential for its regulatory function, but only a minimal amount of ATP hydrolysis by Mcm2 is required for this role.

Mcm3/5 does not restore the activity of Mcm2_{K549A}

DNA unwinding by Mcm4/6/7 is also inhibited by Mcm3/5 in *S. pombe*¹¹ and humans.¹⁵ In these cases, Mcm2 and Mcm3/5 appear to function independently.¹¹ To address this question with the *S. cerevisiae* proteins, we examined the ability of Mcm3/5 to inhibit DNA unwinding by Mcm4/6/7 in the presence and in the absence of wild-type and mutant Mcm2. We found that Mcm3/5 inhibits DNA unwinding by Mcm4/6/7, but not to the same extent as the equivalent molar concentration of Mcm2 (assuming an Mcm3/5 dimer and an Mcm2 monomer; Fig. 9a, compare lanes 4 and 6). When both Mcm2 and Mcm3/5 were added to Mcm4/6/7, DNA unwinding was inhibited further (Fig. 9a, lane 7). However, when Mcm2_{K549A} and Mcm3/5 were added to Mcm4/6/7, the extent of Mcm4/6/7 inhibition was the same as that observed for Mcm3/5 on its own (Fig. 9a, lane 8). These results indicate that Mcm3/5 is unable to compensate for the defect in Mcm2_{K549A} and that Mcm3/5 functions independently of Mcm2 to modulate Mcm4/6/7 activity.

P-loop mutations do not affect inhibition by Mcm3/5

We utilized P-loop mutations in Mcm3 and Mcm5 to determine whether the ATP sites of these proteins are important for their ability to inhibit Mcm4/6/7. Mcm3_{K415A}/5 and Mcm3/5_{K422A} were titrated into DNA unwinding assays containing Mcm4/6/7 (Fig. 9b). Both Mcm3/5_{K422A} and Mcm3_{K415A}/5 inhibit DNA unwinding by Mcm4/6/7 to the same extent as wild-type Mcm3/5. This result suggests that ATP binding or hydrolysis is not important for the regulation of Mcm4/6/7 by Mcm3/5. It should be noted that the lysine-to-alanine mutations in Mcm3 and Mcm5 used here are inviable in yeast.²³ These results also suggest that the ADP-mediated inhibition of Mcm4/6/7 is unique to the Mcm2 subunit.

Discussion

By examining the effects of Mcm2 on the activities of Mcm4/6/7, we have shown that Mcm2 has an essential role in regulating DNA binding by Mcm complexes and that a minimal level of ATP hydrolysis by Mcm2 is required for this activity. Specifically, Mcm2

inhibition of ssDNA binding by Mcm4/6/7 occurs in the presence of ATP and ADP, but not with ATP γ S. Inhibition with an Mcm2 mutant that is unable to hydrolyze ATP occurs in the presence of ADP, and not in the presence of ATP. Since the mutant (Mcm2_{K549A}) interacts with Mcm4/6/7 but does not inhibit Mcm4/6/7, interaction alone is not sufficient for Mcm2 to exert its effects. Rather, our results imply that Mcm2 communicates its nucleotide binding status to the other subunits. The altered properties of Mcm2_{K549A} were also observed in Mcm2–7, indicating that our findings are relevant to the Mcm complex *in vivo*. Furthermore, the requirement for only a minimal level of ATP hydrolysis by Mcm2 suggests that Mcm2 plays a regulatory role—rather than direct catalytic role—in DNA unwinding by Mcm2–7.

ATP sites in Mcm proteins

The ATP sites of Mcm2–7 are located at the interface between subunits, with one subunit binding nucleotide and with the other providing a trans-acting catalytic residue, the arginine finger.^{25,28} A study on *Sulfolobus* MCM has indicated that additional trans-acting residues, conserved in eukaryotic Mcm proteins, are important for the function of the helicase.²⁹ Furthermore, structural studies on viral SF3 helicases, E1 from papillomavirus and large T antigen (Tag) from simian virus 40 [also AAA⁺ (ATPase associated with diverse cellular activities) proteins], illustrate that these trans-acting residues undergo conformational changes that are dependent on the nucleotide bound to the neighboring subunit.^{30,31} Interestingly, it has been noted that some trans-acting residues that contact the phosphates of ATP bound to the neighboring subunit are adjacent to β -hairpin structures that interact with ssDNA in the E1 structure and undergo positional changes in response to ATP binding and hydrolysis.^{29,31} Therefore, binding of nucleotide to one Mcm subunit has the potential to affect the conformation and activity of the adjacent subunit through trans interactions of specific residues with the phosphate moieties of the nucleotide. It is therefore reasonable to envision possible mechanisms by which Mcm2 could communicate its nucleotide-bound state to Mcm4/6/7 and other subunits of Mcm2–7 to affect ssDNA binding by these complexes.

DNA binding by Mcm4/6/7

In contrast with our findings, Mcm4/6/7 did not bind circular ssDNA in a previous study.¹⁴ This may be due to differences in the point of addition of the nucleotide, since we have found that incubation of Mcm4/6/7 with nucleotide before DNA interferes with DNA binding (X. Ma, B.E.S., A. Rezvanpour, and M.J.D., in preparation). We also note that there are differences in the ability of ADP to support DNA binding by Mcm4/6/7 in the two studies. These are not easily explained, but we note that the type of assay utilized and the preparation of proteins were not identical in the two studies.

Mechanism of Mcm2 inhibition of Mcm4/6/7

Mcm2 was previously thought to inhibit Mcm4/6/7 by disrupting the oligomeric structure of Mcm4/6/7.^{9,11} Here, we have shown that interaction with Mcm4/6/7 is not sufficient for Mcm2 to exert its effects; thus, the model that Mcm2 acts solely by disrupting an Mcm4/6/7 hexamer is not upheld. Indeed, Mcm2_{K549A} interacts with Mcm4/6/7 to the same extent as wild-type Mcm2, suggesting that similar-sized complexes are formed with mutant and wild-type Mcm2. We have considered the different mechanisms that may account for the ability

of Mcm2 to disrupt ssDNA binding by Mcm4/6/7. Most simply, Mcm2 could compete with Mcm4/6/7 for binding to DNA. However, we detected only very weak ssDNA binding by Mcm2 (consistent with a previous study³²) such that only 1 of 20 ssDNA molecules was bound by Mcm2 under the conditions used in our assays (data not shown). We have also shown that Mcm2 is unable to remove Mcm4/6/7 from DNA once it is bound (Supplementary Information). Therefore, Mcm2 appears to act before or when Mcm4/6/7–ssDNA complexes are formed, whereby interaction of the ADP-bound form of Mcm2 with Mcm4/6/7 results in the conformation of Mcm4/6/7 that is incapable of binding ssDNA.

The inability of Mcm2_{K549A} to inhibit ssDNA binding and the helicase activities of Mcm4/6/7 are likely closely related. However, it is interesting to note that Mcm2 inhibition of DNA binding was not as robust as its inhibition of DNA unwinding. This difference may reflect a disparity in the dynamic ranges of the assays and/or the dilution of protein complexes that occurs in gel-filtration assays. Alternatively, there may be additional mechanisms by which Mcm2 regulates Mcm4/6/7.

The effects of ATP hydrolysis by Mcm2 on DNA binding by Mcm4/6/7 are presumably communicated allosterically. We argue that the effects of ATP hydrolysis by Mcm2 on DNA binding are not communicated through changes in ATP binding and hydrolysis by Mcm4/6/7, since Mcm2_{K549A} inhibits Mcm4/6/7 ATPase activity, but not DNA binding. In addition, Mcm4/6/7 ssDNA binding is only slightly affected by ATP hydrolysis. Rather, ATP hydrolysis by Mcm2 may affect the conformation of one or more subunits in Mcm4/6/7, thereby reducing DNA binding.

Mcm4/6/7 inhibition by Mcm3/5

DNA unwinding by Mcm4/6/7 is also inhibited by Mcm3/5 (this study, Lee and Hurwitz,¹¹ and Sato *et al.*¹⁵). However, in contrast to Mcm2, mutation of the Mcm5 and Mcm3 P-loops did not interfere with their abilities to regulate Mcm4/6/7. Consistent with previous studies, we have shown that Mcm3/5 and Mcm2 act independently. Interestingly, Bochman and Schwacha have shown that the Mcm2 and Mcm3 P-loops are not essential for DNA unwinding by Mcm2–7⁸ even though mutation of the conserved P-loop lysine to alanine in Mcm2 or Mcm3 results in inviability (as does mutation of Mcm5).²³ Our observation that only the Mcm2 P-loop among these three Mcms regulates DNA binding is consistent with other observations that have led to the suggestion that each ATP site within Mcm2–7 contributes differently to the activity of the complex.^{8,23,28,33}

Mcm2 function within Mcm2–7

The ability of Mcm2 to regulate Mcm4/6/7 is coincident with its ability to support yeast growth; thus, the inhibitory role of Mcm2 likely has a role *in vivo*. It is important to note that each of Mcm2–7 is required for DNA unwinding during DNA replication and that Mcm4/6/7 is not sufficient to support DNA replication;^{4,34,35} thus, a biological role for the effects of Mcm2 on Mcm4/6/7 is found within the context of Mcm2–7. We observed that a functional Mcm2 ATP site is required to modulate ssDNA binding by the Mcm2–7 complex. As with the inhibition of Mcm4/6/7 DNA binding, the ATP binding status of Mcm2 is likely relayed to other Mcm subunits within Mcm2–7 to alter DNA binding of the complex. We

can envision Mcm2's effects on DNA binding with Mcm2-7 playing a role *in vivo* in two ways. One is during DNA unwinding, and the other is within the activation process. In general, helicases are thought to function via repeated cycles of DNA binding, translocation, and DNA release.¹⁹ The translocation steps are coupled to ATP binding and hydrolysis, much like the effects of Mcm2 on DNA binding by Mcm4/6/7. Thus, during translocation, Mcm2 inhibition of DNA binding may be important for DNA release. It would therefore follow that mutations that severely impair ATP hydrolysis by Mcm2 would cripple DNA unwinding by Mcm2-7. Interestingly, Mcm2^{KA}-7 retains DNA unwinding activity, albeit at a reduced level compared to wild-type Mcm2-7, unlike similar mutations in all other Mcm subunits, except Mcm3.⁸ Furthermore, Mcm2^{K549R} with very low ATPase activity, compared to wild-type Mcm2, is able to modulate Mcm4/6/7 activity and supports viability. The low catalytic rate of this mutant might suggest that the Mcm2 ATP site is not necessarily required for the catalysis of helicase activity. Alternatively, ATP hydrolysis by Mcm2 may be one of the control points for Mcm2-7 activation. Perhaps ATP hydrolysis by Mcm2 is switched off during Mcm2-7 activation, thereby relieving inhibition by Mcm2. Interestingly, Mcm2 is one of the targets for DDK, a kinase complex that is thought to participate in the activation of Mcm2-7.⁶

Materials and Methods

Materials

The sources of reagents were Sigma Aldrich for ATP, ADP, and ATPγS (99% purity for each); New England BioLabs for molecular biology enzymes; and Integrated DNA Technologies for helicase substrates, unless otherwise indicated. M13mp18 ssDNA was phenol extracted from polyethylene-glycol-precipitated phage³⁶ and is greater than 95% circular ssDNA. Purification of individual Mcm subunits was performed as described previously,²⁵ with the exceptions described below. For Mcm6, the EAH Sepharose step was omitted. Mcm4/6/7 was reconstituted from individual subunits as described in Kaplan *et al.*¹⁰ Mcm2-7 was reconstituted from individual subunits as described previously,²⁵ except that a MonoS column was performed (buffer H [20 mM Hepes-NaOH (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM DTT, and 10% (vol/vol) glycerol]; 20 ml of 0–500 mM NaCl gradient) before the MonoQ column, and the gel-filtration column was omitted. For ATPases, Mcm6 was purified by gel filtration, as described in Davey *et al.*²⁵ Molar concentrations of proteins are expressed as monomer for Mcm2 and Mcm6, dimer for Mcm3/5, and hexamer for Mcm4/6/7 and Mcm2-7.

Plasmids

mcm2^{K549R} was generated by amplifying a portion of the *MCM2* coding region from pET11a-*MCM2*²⁵ using the mutagenic primer 5'-d(CCAGGTACCGCCAGATCTCA)-3' and a second primer, 5'-d(TCGGGCTTTGTTAG)-3', with Elongase polymerase (Invitrogen). A second round of amplification used the megaprimer product³⁷ generated in the first reaction and 5'-d(AATTGTGAGCGGATAACAATCCCCCTC)-3' to amplify the *MCM2* coding region containing the K549R mutation, as well as NdeI and BamHI sites at the 5' and 3' ends. *mcm2^{K549A}* was generated similarly, except that the mutagenic primer was 5'-d(GATCCAGGTACCGCCGCATC)-3'. The mutant PCR products were digested

with BamHI and NdeI and ligated into the same sites of pET24a (Novagen) to yield pET24a-*mcm2*_{K549R} and pET24a-*mcm2*_{K549A}. Mutations were confirmed by sequencing the *MCM2* coding region (Robarts Research Institute DNA Sequencing Facility).

pMD279, for disruption of *MCM2*, is YIplac211,³⁸ with the 349-bp intergenic region upstream of *MCM2*, as well as the *MCM2* coding sequence in which nucleotides 876–2211 were replaced with a 1.3-kb BsaI fragment encoding *HIS3*.

pMD240 allows expression of genes from the *MCM6* promoter in yeast. Nucleotides 120907–122297 of *S. cerevisiae* chromosome VII were amplified using the primers 5'-d(TATATCTGCAGCAACAAGCCTAATTC)-3' and 5'-d(ATAATCTAGACATATGAAAAAACCAGTTTAAACCT). The resulting 1.4-kbp PCR fragment was digested with PstI and XbaI and inserted into the same sites of YCplac111. The 2.8-kbp NdeI-BamHI fragment from each of pET11a-*MCM2*, pET24a-*mcm2*_{K549R}, and pET24a-*mcm2*_{K549A} was inserted into the same sites of pMD240 to yield pMD269, pMD270, and pMD271, respectively.

*mcm5*_{K422A} was generated from pET16b-*MCM5*²⁵ using the mutagenic primer 5'-d(GATCCAGGTACCGCCGCTAGCCAATTTGAAATTTGTG)-3' and a second primer, 5'-d(GCATATAAGGCCTTATCCAGTG)-3'. The resulting 1-kb fragment was digested with KpnI and StuI and then inserted into the same sites of pET16b-*MCM5* to yield pET16b-*mcm5*_{K422A}. The mutation was confirmed by sequencing the *MCM5* open reading frame across the amplified region.

Yeast strains

MCM2 was disrupted in yeast using a two-step gene replacement.³⁹ pMD279 was linearized by digestion with EcoNI and transformed into BY4743 (*MATa/α his3 1/his3 1 leu2 0/leu2 0 lys2 0/LYS2 MET15/met15 0 ura3 0/ura3 0*⁴⁰). Ura⁺ colonies were grown on YPD and then on synthetic minimal media containing 5-FOA to select cells that had lost *URA3*. Colonies that grew on 5-FOA were plated on media lacking histidine, resulting in YMD33. Disruption in one of the *MCM2* loci was confirmed by PCR. After the transformation of YMD33 with pMD269, the strain was sporulated, tetrads were dissected, and Leu⁺, His⁺ spores were selected, yielding YMD46.

To carry out plasmid shuffling,⁴¹ we switched the *LEU2* marker on pMD269 in YMD46 to *URA3* by transforming YMD46 with the XhoI-HpaI fragment of pLU12,⁴² generating YMD54.

Mcm2 purification

Mcm2 proteins were purified as described previously²⁵ with an appended step to remove a nuclease contaminant. Mcm2-containing fractions from the MonoQ step were pooled (approximately 3 ml, 2.7 mg/ml) and dialyzed overnight against buffer H. The dialysate was applied to a 10-ml ssDNA-Sepharose column,⁴³ washed with 100 ml of buffer H, and eluted with 100 ml of 0–500 mM NaCl gradient.

Mcm3^{PK} and Mcm4^{PK}

A PKA recognition motif was fused to Mcm4 by inserting the 3.1-kb NdeI-ClaI fragment from pET11a-*MCM4*²⁵ into the same sites of pHKEp.⁴⁴ Similarly, the 2.9-kb NdeI-BlnI fragment from pET21b-*MCM3*²⁵ was inserted into the same sites of pHKEP.⁴⁴ Mcm4^{PK} purified similarly to untagged Mcm4;²⁵ however, an ssDNA-Sepharose chromatography step was inserted between the heparin agarose and MonoS steps. Pooled fractions from the heparin agarose column (100 ml, 0.2 mg/ml) were applied to a 20-ml ssDNA Sepharose column equilibrated in buffer H. The column was washed with 200 ml of buffer H and eluted with 200 ml of 0–500 mM NaCl gradient. Mcm4^{PK}-containing fractions were dialyzed against buffer H and applied to a MonoS 5/50 GL column (GE Healthcare), as described previously.²⁵

Mcm3^{PK} was purified similarly to tagged Mcm6,²⁵ with the following modifications. The Mcm3^{PK}-containing fractions from the Ni-affinity chromatography column were dialyzed against buffer H and applied to a 15-ml SP Sepharose column (GE Healthcare) equilibrated in buffer H and eluted with a 0–500 mM NaCl gradient in buffer H. Mcm3^{PK}-containing fractions were pooled and dialyzed against buffer A (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2 mM DTT and 10% (v/v) glycerol) then applied to a MonoQ 10/100 GL column (GE Healthcare) and eluted with 80 ml of 0–500 mM NaCl gradient. Both Mcm3^{PK} and Mcm4^{PK} reconstituted similarly to untagged Mcm3 and Mcm4 into Mcm4/6/7 and Mcm2–7, respectively. The activity of Mcm4^{PK}/6/7 was comparable to that of Mcm4/6/7 in DNA unwinding assays, and the activity of Mcm2–7^{3PK} was similar to that of Mcm2–7 in ATPase assays.

ATP hydrolysis

ATP hydrolysis by Mcm2 (mutant and wild type) in the presence and in the absence of Mcm6, as well as Mcm4/6/7 with and without Mcm2, was assayed using thin-layer chromatography (TLC). Each 15- μ l reaction contained 1 mM [γ -³²P]ATP (20 Ci/mmol; Perkin Elmer Life Sciences), 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, and 2 mM DTT, as well as 1 μ M each of Mcm2 (wild type or mutant) and/or Mcm6 for Mcm2/6 analysis or 200 nM Mcm4/6/7 and/or 400 nM Mcm2 for Mcm4/6/7 analysis. At the indicated times, 2 μ l of each reaction in the Mcm2/6 analysis was removed and quenched with 2 μ l of 50 mM EDTA (pH 8). Mcm4/6/7 analyses were performed over 90 min, with 30-min intervals. One microliter was spotted onto polyethyleneimine cellulose TLC sheets (EM Science), which were developed in 0.6 M potassium phosphate (pH 3.4) for 10 min. The dried TLC sheets were exposed to a PhosphorStorage screen before scanning with a Storm 860 scanner (GE Healthcare). The “volume” of the spots corresponding to P_i and ATP was used to determine the extent of hydrolysis. A background volume was subtracted, and the molar concentration of P_i produced was determined by: $10^3 \times (\text{volume P}_i) / (\text{volume P}_i + \text{volume ATP})$. To calculate the ATP hydrolysis rates of the Mcm2/6 complexes, we subtracted the background levels of hydrolysis by individual subunits. For Mcm4/6/7+Mcm2 reactions, the background levels of hydrolysis by Mcm2 were subtracted. The rate of ATP hydrolysis was determined by the linear regression of plots of time $\times$ pmol P_i/pmol protein using Prism 5 software (GraphPad Software). The rates are the means of three replicate experiments.

DNA unwinding

DNA unwinding measurements were performed with a DNA substrate containing 30 nucleotides of duplex, with 60 nucleotides of ssDNA on one strand and a 5' biotin on the other strand, as described by Kaplan *et al.*¹⁰ Primer 2T 5'-d(ATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-(T)₆₀)-3' was 5' end labeled with ³²P and annealed to 1B 5'-biotin-d(GACGCTGCCGAATTCTGGCTTGCTAGGACAT)-3' by boiling for 2 min in 10 mM Tris-HCl (pH 8.5), 30 mM sodium citrate, and 300 mM NaCl and then by slow cooling to room temperature. Each reaction (6 µl) contained 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 100 µM EDTA, 5 mM DTT, 5 mM ATP, 67 nM streptavidin, 1 nM substrate with 100 nM or 200 nM Mcm4/6/7 (as indicated), and the indicated concentrations of Mcm2 (mutant or wild type) and Mcm3/5 (mutant or wild type). The mixtures were incubated at 37 °C for 10 min, and then unwinding was stopped by the addition of 1 µl of Proteinase K (10 mg/ml) for 2 min at 37 °C, followed by the addition of 1.5 µl of 20 mM EDTA (pH 8.0), 1% SDS, 0.02% bromophenol blue, 5% (vol/vol) glycerol, and 0.02% xylene cyanol FF. The samples were analyzed by native (Tris-borate-EDTA) PAGE (8%), and dried gels were exposed to PhosphorStorage screens before scanning with a Storm 860 scanner (GE Healthcare). The volumes of the bands corresponding to ssDNA product and dsDNA substrate, as well as a background volume, were used to calculate the extent of DNA unwinding after subtracting the background volume: % DNA unwound = volume ssDNA/(volume ssDNA+volume dsDNA)×100.

Affinity chromatography

Mcm4/6/7 (600 nM) was incubated on ice for 15 min with or without equimolar Mcm2 or Mcm2_{K549A} in 100 µl of 5 mM imidazole, 20 mM Tris-HCl (pH 7.9), 5 mM ATP, and 100 mM NaCl. Forty microliters of HIS-Select Nickel Affinity resin (50% slurry in the same buffer; Sigma Aldrich) was added, and the mixtures were incubated for a further 10 min. Resin was pelleted by brief centrifugation at 1000 rpm in a table-top centrifuge. Approximately 40% of the input protein bound to the resin. The supernatant was removed, and the resin was washed five times with 100 µl of 5 mM imidazole, 20 mM Tris-HCl (pH 7.9), 5 mM ATP, and 350 mM NaCl, and eluted with 20 µl of SDS loading dye. Ten percent of the load, 8% of the wash, and 40% of the eluate were analyzed by SDS-PAGE (6%) stained with Coomassie brilliant blue R-250. In addition, the eluate was probed with anti-Mcm2 antibody (sc6680; Santa Cruz Biotechnology) using rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma Aldrich) and chemiluminescence (Pierce) to detect the signal.

Labeling of Mcm4^{PK}/6/7 and Mcm2-7^{3PK}

Mcm4^{PK}/6/7 (100 pmol) or Mcm2-7^{3PK} (50 pmol) was radiolabeled by incubation with 50 µCi of [³²P]ATP (3000 Ci/mmol; Perkin Elmer Life Sciences), 12.5–25 U of PKA catalytic subunit (from bovine heart; Sigma Aldrich), 20 mM Tris-HCl (pH 8.0), and 20 mM magnesium acetate for 1 h at 21 °C. Labeled protein was separated from [³²P] ATP using a 5-ml Sephadex G-25 column (GE Healthcare) equilibrated in buffer A containing 100 mM NaCl and 50 µg/ml bovine serum albumin. The labeled complexes typically had specific

activities of 1–1.5 $\mu\text{Ci}/\text{pmol}$ complex. Greater than 99% of unincorporated [^{32}P]ATP was removed by gel filtration.

DNA binding

DNA binding assays were performed in 100 μl of buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 100 μM EDTA, and 2 mM DTT, as well as 5 mM ATP, ADP, or ATP γS , as indicated. When added, each assay contained 10 pmol of Mcm2 or Mcm2_{K549A}, 1 pmol of Mcm4^{PK}/6/7, and 100 fmol of Mcm2–7^{3PK} and/or Mcm2_{K549A}–7^{3PK}. Proteins, buffer, and nucleotide were incubated on ice for 10 min before the addition of M13mp18 ssDNA [2 pmol (as M13 DNA molecule) in the Mcm4/6/7 assays and the indicated amounts for Mcm2–7 assays in 1.5 μl], and the mixtures were incubated for 10 min at 37 °C. Two microliters of the reaction was removed for liquid scintillation counting (load), and the remainder was applied to a 5-ml 4% plain agarose bead column (Agarose Bead Technologies) equilibrated in buffer A containing 100 mM NaCl, 10 mM magnesium acetate, and 40 $\mu\text{g}/\text{ml}$ bovine serum albumin. The column was eluted with the same buffer, and eight drop fractions (~200 μl) were collected. Twenty microliters of the indicated fractions was analyzed by liquid scintillation counting. The amount (in fmol) of labeled Mcm4^{PK}/6/7 or Mcm2–7^{3PK} in each fraction was determined by: [cpm fraction (fraction volume/fraction volume counted)]/[(cpm load/fmol in load)×(load volume/load volume counted)]. A representative graph from at least three replicates of each experiment is shown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Mcm	minichromosome maintenance
DDK	Dbf4-dependent kinase
Cdc	cell division control
ssDNA	single-stranded DNA
dsDNA	double-stranded DNA
PKA	protein kinase A
ATPγS	adenosine 5'-O-(3-thio)triphosphate
5-FOA	5-fluoroorotic acid

EDTA	ethylenediaminetetraacetic acid
TLC	thin-layer chromatography

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Fig. 1.

Mutations at the Mcm2/6 interface reduce ATP hydrolysis by Mcm2/6. (a) A schematic of the Mcm2/6 ATPase site, located at the subunit interface, is shown. The P-loop of Mcm2, which includes the invariant lysine residue (residue 549), is indicated. “SRF” represents conserved residues in Mcm6 that are likely required for ATP hydrolysis by Mcm2.²⁵ (b) ATP hydrolysis by Mcm2 (“2WT,” filled square), Mcm2_{K549A} (“2KA,” filled triangle), Mcm6 (“6WT,” filled diamond), Mcm6_{R708A} (“6RA,” empty triangle), Mcm2+Mcm6 (“2WT/6WT,” empty square), Mcm2_{K549A}+Mcm6 (“2KA/6WT,” empty circle), and Mcm2+Mcm6_{R708A} (“2WT/6RA,” empty diamond) was measured. Reactions were quenched at 30 min, 60 min, and 90 min, and micromolars of P_i produced at each time point were determined. The reactions were performed in triplicate, and the mean and standard deviation of each time point were plotted. Standard deviation bars are often eclipsed by the symbols. Addition of ssDNA did not affect ATP hydrolysis (data not shown), as previously observed.^{23,25} (c) The rates less the background hydrolysis of individual proteins from (b) are shown with the standard deviation.

Fig. 2. Interaction of Mcm2 with Mcm4/6/7. (a) Affinity-chromatography-based assay for Mcm4/6/7 interaction with Mcm2. Mcm4/6/7 and/or Mcm2 (mutant or wild type) was mixed and then bound to nickel resin. The resin was washed and then eluted with SDS loading dye. Ten percent of the load (“L”), 8% of the wash (“W”), and 40% of the elution (“E”) for each experiment are shown. Mcm2 flows through the column when analyzed alone (data not shown). The Mcm4/6/7-alone experiment (lanes 1–3) was analyzed on a separate gel. (b) Western blot analysis of the elutions from (a). Anti-Mcm2 antibody was used to confirm the presence or the absence of Mcm2 (wild type or mutant) in the elutions of the experiments in (a). Lanes 1 and 2 are controls with known amounts of Mcm2; lanes 3 through 6 are the elutions of affinity resin experiments. The input protein(s) is shown above the lane. The migration of size markers through the gel is shown on the left.

Fig. 3.

Effect of Mcm2 on ATP hydrolysis by Mcm4/6/7. ATP hydrolysis was measured with Mcm4/6/7 in the presence and in the absence of Mcm2 or Mcm2_{K549A}, as described in Materials and Methods. Three separate experiments were performed, and the rates of hydrolysis were determined (less background hydrolysis by Mcm2 or Mcm2_{K549A}) and plotted with the standard error of the mean. Addition of ssDNA did not affect activity (data not shown).

Fig. 4. Mcm4/6/7 DNA unwinding inhibition by wild-type and mutant Mcm2. (a) DNA unwinding in the presence of mutant and wild-type Mcm2 was examined. The indicated concentrations of wild-type Mcm2 ("2WT") or Mcm2_{K549A} ("2KA") were mixed with 200 nM Mcm4/6/7 and assayed for DNA unwinding. (b) The percentage of DNA unwinding was calculated from four replicates and the amount of ssDNA produced from total DNA.

Fig. 5.

Binding of Mcm4/6/7 to ssDNA. DNA binding was measured in a gel-filtration-based assay, as described in Materials and Methods. Plots indicate the amount of Mcm4^{PK}/6/7 in each fraction, as determined by scintillation counting of ³²P-labeled Mcm4^{PK}. (a) The binding of Mcm4^{PK}/6/7 to ssDNA (square) and dsDNA (circle) in the presence of ATP is shown. Mcm4^{PK}/6/7 in the presence of ATP, but in the absence of DNA, is also indicated (triangle). (b) Mcm4^{PK}/6/7 binding to ssDNA in the presence (square) and in the absence (circle) of 5 mM ATP. (c) Comparison of Mcm4^{PK}/6/7 binding to ssDNA with ATP (square) or ADP (circle), and (d) with ATP (square) or ATP γ S (circle). Note that Mcm4/6/7 binding to ssDNA was examined using a range of protein and DNA concentrations, and representative assays are shown to demonstrate the effect of Mcm2 on Mcm4/6/7 interaction with ssDNA (as described below). The relative bindings of Mcm4/6/7 to ssDNA with ATP, ADP, and ATP γ S were similar under all the conditions tested.

Fig. 6.

Mcm2 inhibits ssDNA binding by Mcm4/6/7. DNA binding was measured in a gel-filtration-based assay. Plots indicate the amount of Mcm4^{PK}/6/7 in each fraction. (a) Mcm4^{PK}/6/7 binding to ssDNA in the absence (square) and in the presence of wild-type (circle) or mutant (triangle) Mcm2 with 5 mM ATP. (b) Mcm4^{PK}/6/7 binding to ssDNA in the presence (circle) and in the absence (square) of 10 pmol of Mcm2 with (b) 5 mM ATP γ S, and (c) Mcm4^{PK}/6/7 binding to ssDNA in the absence (square) and in the presence of wild-type (circle) or mutant (triangle) Mcm2 with 5 mM ADP. (d) Quantitative Western blot analysis of Mcm2 (mutant or wild type) in DNA binding experiments with 5 mM ATP. The amount of Mcm2 determined from the blots, as well as the amount of Mcm4^{PK}/6/7 determined by scintillation counting in the peak fraction (fr 11) from the experiments in (d) and (e), is shown. The amount of Mcm2 that binds to M13 in the absence of Mcm4/6/7 was subtracted.

Fig. 7.

DNA binding by Mcm2_{K549A}-7 is enhanced compared to DNA binding by Mcm2-7. (a) Mcm2-7^{3PK} and Mcm2_{K549A}-7^{3PK} were reconstituted from pure subunits. (a and b) DNA binding of Mcm2-7 complexes was measured using gel filtration, as described in Materials and Methods. Binding of Mcm2-7^{3PK} (a) and Mcm2_{K549A}-7^{3PK} (b) in the absence of nucleotide was measured in the presence (square) and in the absence (circle) of ssDNA. (c–e) DNA binding by Mcm2-7^{3PK} (square) or Mcm2_{K549A}-7 (circle) was measured with either 100 fmol (c), 75 fmol (d), or 50 fmol (e) of ssDNA in the presence of ATP. (f) The amount of DNA binding by Mcm2-7^{3PK} or Mcm2_{K549A}-7^{3PK} was quantified by summing the amount of Mcm2-7^{3PK} found in the peak fractions (fractions 10 through 15). The amount of Mcm2-7^{3PK} in the peak fractions for three replicates each at DNA concentrations of 50 fmol, 75 fmol, and 100 fmol, less the amount of protein found in the early fractions in the absence of ssDNA, was quantified and plotted.

Fig. 8.

Minimal ATP hydrolysis by Mcm2 supports yeast cell viability and Mcm2. (a) ATP hydrolysis by Mcm2+Mcm6 (“2WT/6WT”) and Mcm2_{K549R}+Mcm6 (“2KR/6WT”) was measured. Reactions were quenched at 30 min, 60 min, and 90 min, and micromolars of P_i were produced at each time point. Shown are the rates of ATP hydrolysis less the background hydrolysis of Mcm2, Mcm2_{K549R}, and Mcm6, with the standard deviation. (b) The viabilities of wild-type *MCM2* (2WT), *mcm2_{K549R}* (2KR), and *mcm2_{K549A}* (2KA) were determined by plasmid shuffling on media containing 5-FOA. “V” indicates a vector control with no *MCM2* insert. Growth of shuffled strains containing *MCM2* (WT) or *mcm2_{K549R}* (KR) at 16 °C, 30 °C, and 35 °C is shown on the right. (c) DNA unwinding in the presence of mutant and wild-type Mcm2 was examined. Wild-type Mcm2, Mcm2_{K549R}, or Mcm2_{K549A} was mixed with equimolar (200 nM) Mcm4/6/7 and assayed for DNA unwinding. Shown is a representative PhosphorImager scan. Lanes 1 and 2 are the dsDNA substrate and ssDNA product, respectively, and their migrations through the gel are shown on the right. The substrate includes a 5′ biotin on the top strand, as indicated by the black sphere. The proteins added to each assay are indicated at the top of the gel, and lane numbers are shown underneath.

Fig. 9.

Inhibition of Mcm4/6/7 by Mcm3/5. (a) Mcm2 and/or Mcm3/5 inhibition of DNA unwinding by Mcm4/6/7 was analyzed. Mcm2, Mcm3/5, and Mcm2_{K549A} (100 nM) were mixed as indicated with 100 nM Mcm4/6/7, and DNA unwinding was assayed. The migrations of dsDNA substrate and ssDNA product are indicated to the right of the gel. (b) DNA unwinding by Mcm4/6/7 was examined in the presence or in the absence of increasing amounts of Mcm3/5 (filled circle), Mcm3_{K415A}/5 (empty circle), or Mcm3/5_{K422A} (square). The means of three experiments are plotted.