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Short Article

The Mcm2-7 Complex Has In Vitro Helicase Activity

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SUMMARY

Helicases unwind duplex DNA ahead of the polymerases at the replication fork. However, the identity of the eukaryotic replicative helicase has been controversial; in vivo studies implicate the ring-shaped heterohexameric Mcm2-7 complex, although only a specific subset of Mcm subunits (Mcm467) unwind DNA in vitro. To address this discrepancy, we have compared both Mcm assemblies and find that they differ in their linear single-stranded DNA association rate and their ability to bind circular single-stranded DNA. These differences depend upon the Mcm2/5 interface, which we hypothesize serves as an ATP-dependent "gate" within Mcm2-7. Importantly, we find that reaction conditions that putatively close the Mcm2-7 "gate" reconstitute Mcm2-7 helicase activity. Unlike Mcm467, Mcm2-7 helicase activity is strongly anion dependent. Our results show that purified Mcm2-7 acts as a helicase, provides functional evidence of a Mcm2/5 gate, and lays the foundation for future mechanistic studies of this critical factor.

INTRODUCTION

Replicative helicases are motor proteins that use ATP binding and hydrolysis to unwind duplex DNA into single-stranded substrates for DNA polymerase. In eukaryotes, in vivo observations implicate the Mcm2-7 complex as the replicative helicase (reviewed in Bell and Dutta, 2002). Mcm2-7 is a heterohexamer of six essential but nonidentical subunits (numbered 2 through 7) that are each AAA$^+$ ATPases (Forsburg, 2004). Although little structural information exists for this complex, it is known to be toroidal (Adachi et al., 1997; Bochman and Schwacha, 2007; Sato et al., 2000), and similar to other AAA$^+$ proteins, it has ATPase active sites at dimer interfaces with one subunit contributing the Walker A motif and the adjoining subunit contributing an essential arginine (Davey et al., 2003; Moreau et al., 2007).

Despite these in vivo observations, Mcm2-7 has been reported to lack in vitro helicase activity (Bochman and Schwacha, 2007; Davey et al., 2003; Schwacha and Bell, 2001). Interestingly, both an archaeal Mcm complex (reviewed in Kelman and White, 2005) and an alternative hexameric Mcm complex containing only three of the six eukaryotic Mcm subunits (Mcm4, -6, and -7; the Mcm467 complex) have DNA-unwinding activity (Ishimi, 1997; Kaplan et al., 2003; Lee and Hurwitz, 2001). Recently, a larger complex containing Mcm2-7, GINS, and Cdc45 has been isolated and shown to have in vitro helicase activity (CMG complex; Moyer et al., 2006).

Here, we report the reconstitution of Mcm2-7 helicase activity. Through a detailed biochemical comparison of the Mcm2-7 and Mcm467 complexes, we learn that these Mcm assemblies differ in their ability to bind circular ssDNA. The source of this difference is an ATPase active site composed of Mcm2 and Mcm5 (the Mcm2/5 active site). We hypothesize that the Mcm2/5 active site functions as an ATP-dependent gap or "gate" in the toroid and find that the biochemical activities of Mcm2-7, but not Mcm467, are anion dependent. Moreover, we discovered that reaction conditions that preferentially reduce the ability of the complex to bind circular ssDNA (close the "gate") reconstitute Mcm2-7 helicase activity. These findings provide direct biochemical evidence that the Mcm2-7 complex is a DNA helicase, predict an in vivo role for the Mcm2/5 active site in Mcm2-7 loading or activation, and remove a major biochemical obstacle toward mechanistic studies of the Mcm2-7 complex.

RESULTS

Mcm2-7 Binds Circular ssDNA with Higher Affinity than Mcm467

Both Mcm467 and Mcm2-7 form toroidal hexamers and likely bind ssDNA within their central channel (Bochman and Schwacha, 2007). To test the topological consequences of the toroidal structure, competition experiments were conducted to compare their ability to bind linear or circular M13 ssDNA (Figure S1A available online). Using an established filter-binding assay utilizing a radiolabeled linear oligonucleotide probe (Bochman and Schwacha, 2007), we find that although unlabeled linear ssDNA effectively competes for binding by either Mcm complex, circular ssDNA competes well with Mcm2-7 but poorly with Mcm467 (Figure 1A). Similar results were obtained using circular and linear phage ϕX174 ssDNA as a competitor and several different radiolabeled oligonucleotides (data not shown). This result suggests that Mcm467 topologically excludes circular ssDNA from its central channel, whereas Mcm2-7 either transiently opens its ring structure or binds circular ssDNA on an external surface.

ATP binding stimulates the oligomerization of some helicases (e.g., Reynisdottir et al., 1993). To examine a potential role for ATP in closure of the Mcm2-7 toroid, we modified our competition assay into an order-of-addition assay to test if ATP preincubation of Mcm2-7 decreases its ability to bind circular ssDNA (Figure 1B). To simplify experimental interpretation, the nonhydrolyzable nucleotide analog ATPγS was used. This assay was conducted in two steps (Mcm circularization assay, Figure 1C). The Mcms are first incubated for an hour with either ATPγS in

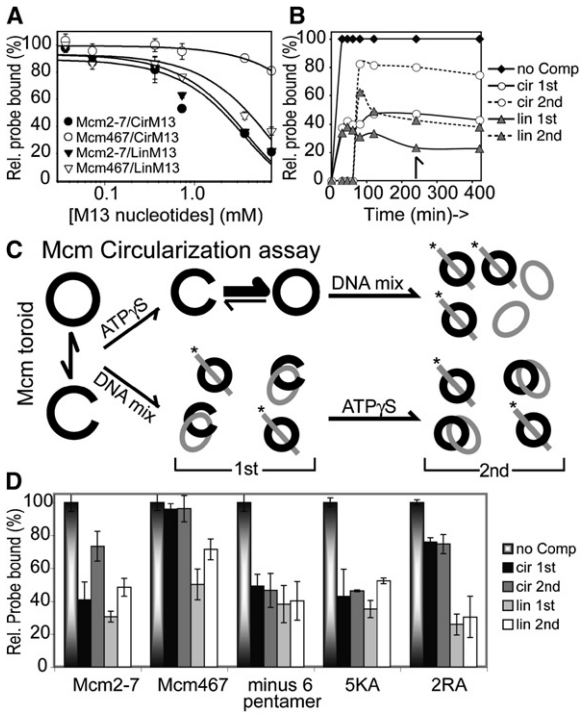

Figure 1. Effects of ssDNA Topology on Mcm Binding

(A) Radiolabeled oligo no. 826 binding in the absence of competitor ssDNA is 100%; other values represent binding competition in the presence of the indicated amounts of unlabeled M13 ssDNA (cir, circular; lin, linear; mM nucleotides). The IC_{50} values are as follows: Mcm2-7 with circular ssDNA, 1.6 ± 4 mM; Mcm2-7 with linear ssDNA, 1.8 ± 3 mM; and Mcm467 with linear ssDNA 3.8 ± 0.6 mM. Mcm467 with circular ssDNA has an affinity too low to accurately measure. From extrapolation of the available data points, we estimate its IC_{50} at > 32 ± 8 mM.

(B) Time course of Mcm2-7 circularization. 100% represents probe binding in the absence of ssDNA competitor. The 240 min values were used in (D).

(C) Circularization assay. Intact or split circles are Mcm hexamers. DNA mix is radiolabeled oligo no. 455 (gray line with asterisk) and either linear (data not shown) or circular (gray oval) unlabeled M13 competitor. 1st and 2nd refer to the addition order of the DNA mix relative to the ATPγS.

(D) Comparison of circularization assays at t = 240 min for the indicated wild-type and mutant Mcm preparations. Conditions were identical to (B).

the absence of DNA or with a mixture of radiolabeled linear ssDNA probe and unlabeled competitor ssDNA (either linear or circular single-stranded M13) in the absence of ATPγS. In the second step, the complete reaction is reconstituted (i.e., the ssDNA mixture is added to the ATPγS preincubation, or ATPγS is added to the ssDNA preincubation), and Mcm association with the radiolabeled oligonucleotide is quantified over time by filter binding. Since probe binding and competition are inversely related, quantitation of probe binding provides an assessment of competition. For Mcm2-7, ATPγS preincubation reduces the efficiency of circular ssDNA as a competitor (Figure 1B, cir 1st versus cir 2nd) with negligible effects on the ability of linear ssDNA to compete (Figure 1B, lin 1st versus lin 2nd). The ability of ATPγS preincubation to block circular ssDNA competition/binding is remarkably stable (≥7 hr, Figure 1B), and qualitatively similar results were obtained when ATP was substituted for ATPγS (data not shown). This result indicates that ATP occupancy of one or more active sites inhibits binding to circular ssDNA and is consistent with a model in which ATP stimulates closure of the Mcm2-7 ring. To simplify our presentation, we will subsequentially refer to circular ssDNA competition as circular ssDNA binding.

Similar circularization experiments were conducted with Mcm467 (Figure S2A). To facilitate comparison, results from the 240 min time point are shown (Figure 1D). In sharp contrast to Mcm2-7, circular ssDNA binds poorly regardless of ATP addition order. To determine if a complete Mcm complex is required for this effect, we examined a pentameric Mcm complex lacking Mcm6 (minus 6 pentamer) that has a predominately open-ring structure (Figure S2C). Results from the circularization assay (Figure S2B) indicate that this complex binds both circular and linear ssDNA equally and independently of ATPγS addition order (Figure 1D), consistent with this assay providing a read-out for Mcm complex topology.

Taken together, a parsimonious interpretation of these results would be that circular ssDNA binds within the central channel of Mcm complexes and that the Mcm2-7 toroid contains a gap ("gate") that Mcm467 lacks. Since the main differences between these two complexes are the Mcm2, -3, and -5 subunits, the simplest hypothesis is that the gate involves one or more of these subunits. Although other interpretations are possible, this hypothesis provides the motivation for many of the following experiments.

The Mcm2/5 ATPase Active Site Mediates Circular ssDNA Interactions in Mcm2-7

Since Mcm ATPase active sites are formed at dimer interfaces (Davey et al., 2003), the relationship between ATP addition order and circular ssDNA binding would be explained if a specific ATPase active site corresponds to the Mcm2-7 gate. To test this possibility, we examined mutant Mcm2-7 complexes containing a single subunit with a lysine to alanine substitution (KA alleles) in the Walker A box (Schwacha and Bell, 2001) in the company of five wild-type subunits. Since the KA mutations in Mcm4 and Mcm7 interfere with ATP-dependent ssDNA binding (Bochman and Schwacha, 2007), their analysis was not pursued. Circularization assays using the Mcm2KA, 3KA, or 6KA complexes yielded results similar to wild-type Mcm2-7, with ATPγS preincubation causing a reduction in circular ssDNA binding (Figure S3A). However, similar to the minus 6 Mcm pentamer, the Mcm5KA complex displays good binding to circular ssDNA independent of ATP addition (Figure 1D).

To further characterize the role of Mcm5 in circular DNA binding, additional Mcm mutations were examined. Subunit association studies suggest that Mcm2 and Mcm5 form an ATPase active site, with Mcm5 contributing the Walker A motif and Mcm2 contributing the essential arginine (Davey et al., 2003). Alanine substitutions were made in the putative arginine finger of each subunit (RA alleles), and a set of six Mcm2-7 complexes analogous to the KA set were expressed and purified. The characterization of these alleles will be presented elsewhere (M.L.B., S.P. Bell, and A.S., unpublished data); however, none of these changes substantially interferes with the in vitro oligomerization or stability of the Mcm2-7 complex (Figures S1B and S1E), and only the Mcm3RA mutation blocks in vitro ssDNA binding (data

not shown). Although the Mcm4RA, 5RA, 6RA, and 7RA complexes exhibit qualitatively wild-type Mcm2-7 activity in the circularization assay (Figure S3B), the Mcm2RA complex binds circular ssDNA equally and independently of ATP addition order (Figure 1D). However, in contrast to the Mcm5KA complex but similar to the Mcm467 complex, the 2RA complex binds circular ssDNA poorly under both conditions, suggesting that this mutant complex forms a closed toroid even in the absence of ATPγS. Taken together, these results implicate ATP binding by the Mcm2/5 dimer interface as particularly important for the circular ssDNA binding.

Relationship between Mcm2-7 Circularization and ssDNA Association Rate

We have previously reported another Mcm2-7 activity that varies with ATP preincubation (Bochman and Schwacha, 2007). Mcm467 binds ssDNA >5-fold more quickly than Mcm2-7. However, preincubation of Mcm2-7 with ATP increases its association rate to Mcm467 levels, (Bochman and Schwacha, 2007, summarized in Figure S3C). Moreover, this association rate enhancement is also mediated by the Mcm2/5 active site, since Mcm2-7 complexes with either the Mcm2RA or 5KA mutation bind ssDNA at the same rate as Mcm467 even without ATP preincubation (Bochman and Schwacha, 2007).

The dependence of ssDNA association rate and circular ssDNA binding upon ATP addition order suggests a common mechanism. In support of this conjecture, we find that both activities (i.e., ssDNA association rate enhancement and inability to bind circular ssDNA, Figure 2A) depend upon extensive ATP preincubation (25–30 min) to achieve their maximal effect, consistent with a slow ATP-dependent conformational change within Mcm2-7. Nevertheless, these two activities can be experimentally uncoupled. Whereas the minus 6 pentamer binds circular ssDNA independent of ATP addition order (Figure 1D), its association rate is still enhanced following ATP preincubation (Figure 2B). These results indicate that although an artificial gap in the Mcm complex can substitute for the Mcm2/5 site to facilitate circular ssDNA binding, the association rate activity may be mechanistically distinct.

Activation of the Mcm2-7 Helicase

We considered the possibility that the lack of Mcm2-7 helicase activity is related to the closure of the Mcm2/5 gate. Although preincubating Mcm2-7 with ATP failed to reconstitute helicase activity (data not shown), additional possibilities were considered. We note that the role of ATP in Mcm2-7 gate closure seems contradictory: both ATP preincubation (suggesting a positive role for ATP) and inactivation of the Mcm2/5 ATPase active site (suggesting a negative role for ATP) have similar effects on circular ssDNA binding and association rate. An explanation consistent with our findings would be that each treatment displaces an unknown inhibitor from the Mcm2/5 active site.

Investigations of such an inhibitor lead to the reconstitution of Mcm2-7 helicase activity. One specific possibility was that ADP that either copurified with the complex or was formed under assay conditions inhibits Mcm2-7 helicase activity. Although our helicase assay contains an efficient ATP regenerating system, extremely low residual amounts of ADP might be

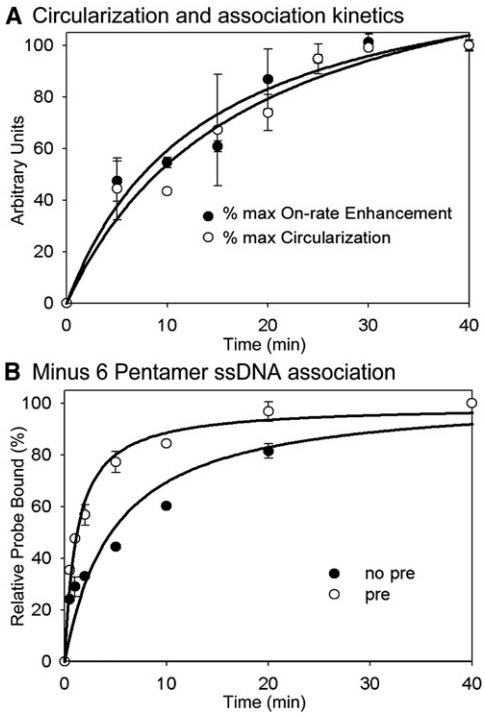

Figure 2. Relationship between Mcm/ssDNA Association Rate and Circularization

(A) Mcm2-7 was preincubated with ATPγS for the indicated times and then added to circularization and association rate assays. 100% corresponds to either the maximum association rate activity observed or the least effective competition with circular ssDNA.

(B) Association rate of the minus 6 pentamer to oligo no. 510 in the absence (no pre) or presence (pre) of ATPγS preincubation.

sufficient to poison Mcm2-7 helicase activity. By increasing the concentration of ATP regeneration components (phosphocreatine and creatine kinase) several fold to further decrease ADP levels, Mcm2-7 helicase activity was uncovered (Figure 3A, compare lanes 4 and 8). This new DNA unwinding activity was both Mcm dependent (blocked by Mcm-specific antibody AS1.1, Figure 3A, lanes 9–11) and ATP dependent (Figure 3A, compare lanes 4 and 12) with a $k_{1/2}$ of 1.8 mM for ATP (data not shown).

Further investigation reveals that Mcm2-7 helicase activity is not inhibited by ADP but by specific ions in the standard assay. We found that the activation of the Mcm2-7 helicase only required the phosphocreatine component of the ATP regenerating system (Figure 3A, compare lane 4 to lanes 5–7), indicating that ATP regeneration is unnecessary for helicase activity. Given the positive effect of phosphocreatine, we investigated if other salts/ions would stimulate this activity. We tested a variety of representative anions, cations, and volume excluders (Figure 3B). Only acetate and glutamate supported Mcm2-7 helicase activity, indicating that unlike many other enzymes, Mcm2-7 helicase activity is anion specific and shows little dependence on the Hofmeister series (reviewed in Record et al., 1978). As potassium glutamate provided the largest stimulation, it was used in subsequent experiments.

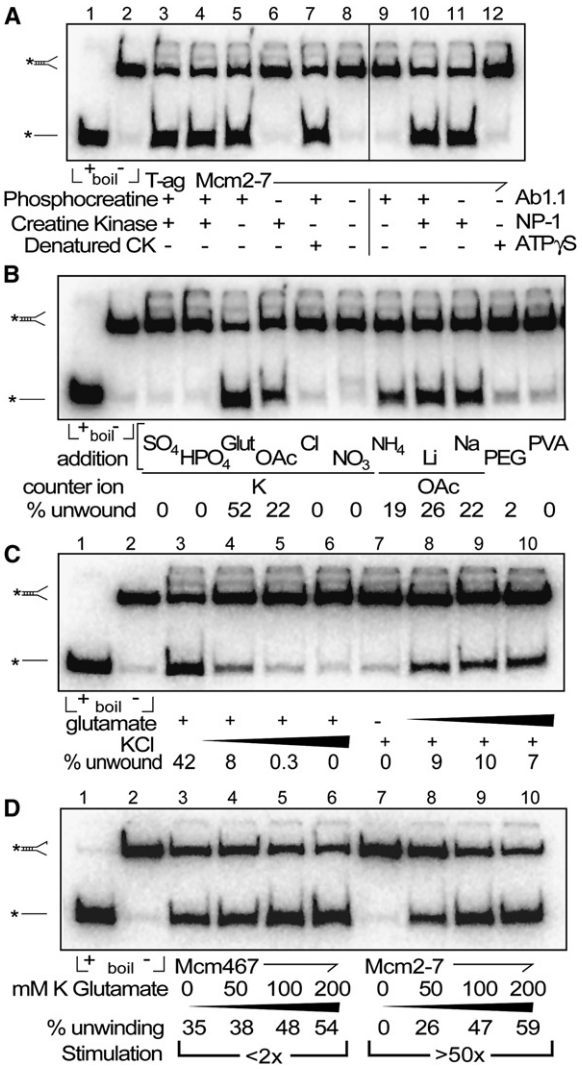

Figure 3. Mcm2-7 Helicase Activity

(A) Phosphocreatine activates Mcm2-7 helicase activity. Lane 1 shows denatured fork substrate (+ boil); lane 2 shows the intact fork (− boil). Lane 3 contains 1 pmol T antigen monomer. Lanes 4–12 contain 400 ng Mcm2-7. In lanes 9–12, and where indicated in lanes 3–8, reactions contain 100 mM phosphocreatine, 100 μg/ml creatine kinase, or both. In lane 7, the ATP regenerating system was boiled (Denatured CK) prior to use. The reactions in lanes 9–12 contain antibody AS1.1, neutralizing peptide (NP), or both as indicated. The reactions in lanes 4 and 12 are equivalent, but 5 mM ATPγS was substituted for ATP in lane 12.

(B) Mcm2-7 helicase activity is anion dependent. Standard helicase reactions containing 400 ng Mcm2-7 were supplemented with either the indicated salt (100 mM; Glut, glutamate; OAc, acetate) or volume excluder (1% [w/v]; PEG, polyethylene glycol 8000, PVA, polyvinyl alcohol).

(C) Competition between glutamate and chloride for Mcm2-7 helicase activity. Reactions contained 50 mM of either potassium glutamate (lanes 3–6) or potassium chloride (lanes 7–10) and 0, 50, 100, or 200 mM of the corresponding salt as noted.

(D) Helicase activity of Mcm467 and Mcm2-7. Reactions contained standard helicase buffer with 5 mM phosphocreatine and 20 μg/ml creatine kinase and contained either no Mcm (lanes 1 and 2), 400 ng Mcm467 (lanes 3–6), or 400 ng Mcm2-7 (lanes 7–10) and were supplemented with potassium glutamate as indicated.

To address if certain anions inhibit helicase activity rather than fail to support it, assays containing mixtures of glutamate and chloride were conducted (Figure 3C). Although 50 mM potassium glutamate supports helicase activity, addition of modest concentrations of potassium chloride inhibits the activity. Conversely, although 50 mM potassium chloride blocks helicase activity, addition of sufficient glutamate partially restores helicase activity. These results indicate that chloride and glutamate have opposite effects on Mcm2-7 helicase activity, consistent with the hypothesis that glutamate displaces inhibitory ions (e.g., chloride) from Mcm2-7.

Since helicase activity has not been previously observed from Mcm2-7, its activity is presumably more sensitive to ionic conditions than Mcm467. To test this hypothesis, the ability of glutamate to stimulate helicase activity from both Mcm2-7 and Mcm467 was compared (Figure 3D). Although glutamate stimulates Mcm467 helicase activity <2-fold over a range of 0–200 mM, Mcm2-7 helicase activity is stimulated at least 50-fold over the same concentration range. This result accounts for the previous inability to detect Mcm2-7 helicase activity and suggests that the basis of this glutamate stimulation involves Mcm2, -3, and/or -5.

Mcm2-7 Helicase Activity Correlates with Reduced Ability to Bind Circular ssDNA

To understand why glutamate facilitates Mcm2-7 helicase activity, we examined its effects on ssDNA binding. The reconstitution of helicase activity does not correspond to an increased affinity for ssDNA, since glutamate does not alter the ssDNA binding constant of Mcm2-7 (Figure 4A). However, preincubation of Mcm2-7 with glutamate generates results similar to ATP preincubation: it decreases the ability of Mcm2-7 to bind circular, but not linear, ssDNA (Figure 4B) and enhances the apparent association rate between Mcm2-7 and linear ssDNA (Figure 4C). Similar to ATP preincubation, the maximal effect of glutamate preincubation on either activity requires about 25–30 min (data not shown). Moreover, both glutamate and ATP preincubation of any of the different Mcm2-7 KA or RA mutant complexes produces qualitatively similar results for both the ssDNA association rate and circularization (Figures 4B and 4D; Figure S4A). Complexes containing the Mcm5KA or 2RA subunits were immune to glutamate preincubation, whereas Mcm2-7 complexes containing the other mutant subunits demonstrated an increase in their apparent association rate and circularization similar to those observed following ATP preincubation (Figures 4B and 4D; Figure S4B). However, even upon increasing the concentration of glutamate (data not shown), ATP preincubation is more effective than glutamate preincubation with wild-type Mcm2-7, suggesting that these two ions may function through slightly different but overlapping mechanisms. These results suggest that like ATP preincubation, glutamate preincubation acts through the Mcm2/5 active site and functions to close the gate in the Mcm2-7 toroid.

Mcm2-7 Helicase Activity Requires at Least Five of the Six Mcm Subunits

Although our results suggest that glutamate stimulates the Mcm2-7 helicase through closure of the Mcm2/5 gate, other trivial explanations are possible. One is that glutamate dissociates

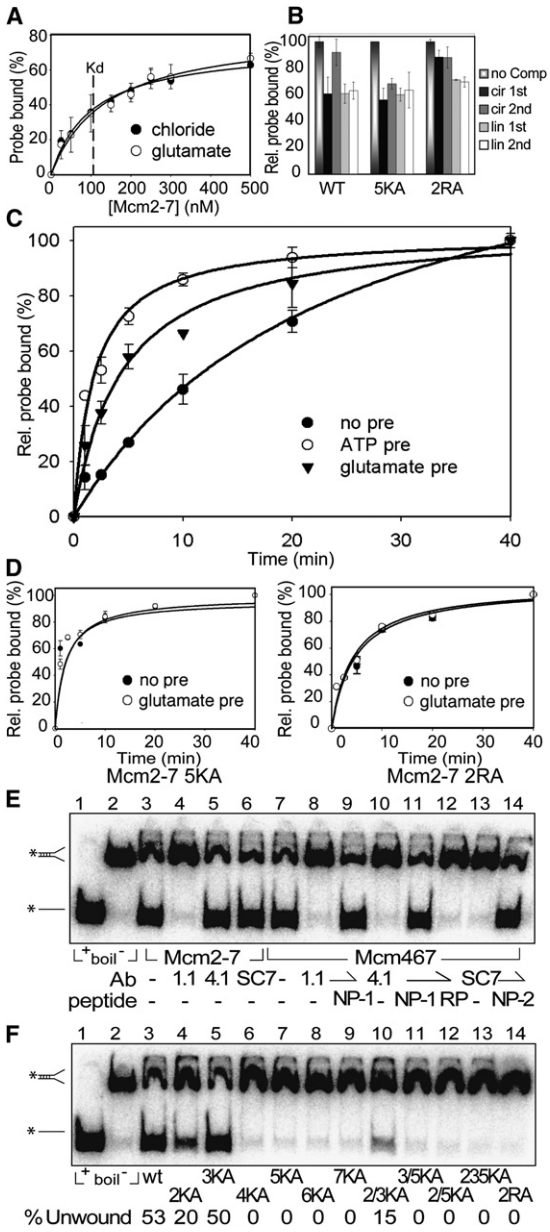

Figure 4. The Effects of Glutamate and Mcm Mutations on Mcm2-7 Helicase Activity

(A) Glutamate does not affect the affinity of Mcm2-7 for ssDNA. Standard filter-binding reactions were carried out in buffer B2 (modified to lack potassium chloride) supplemented with either 100 mM potassium chloride or 100 mM potassium glutamate using radiolabeled oligo no. 826. The K_d in the presence of chloride was 100 ± 14 nM while the K_d in the presence of glutamate was 130 ± 22 nM.

(B) Glutamate preincubation stimulates Mcm2-7 ring closure. Reactions were set up essentially as in Figure 1C, but the ATPγS preincubation was replaced by preincubation with 100 mM potassium glutamate; ATPγS was added to the reactions in all cases after 1 hr of either glutamate or ssDNA preincubation. The 120 min time point is shown for each complex (1 hr preincubation plus 1 hr with all reaction components).

(C) Glutamate preincubation stimulates Mcm2-7 on rate. Reactions were set up essentially as in Figure 2B, but the ATPγS preincubation was replaced by preincubation with 100 mM potassium glutamate. Radiolabeled oligo no. 826 was used.

(D) Association rates of Mcm2-7 complexes containing either Mcm5KA or 2RA in the absence (no pre) or presence (glutamate pre) of glutamate preincubation.

(E) Antibody inhibition of the Mcm467 helicase. NP-1 is the neutralizing peptide for AS1.1 and AS4.1, NP-2 is the neutralizing peptide for SC-7, and RP is a sequence-randomized version of NP-1.

(F) Mcm2-7 Walker A box mutations inhibit helicase activity. Reactions contain 400 ng of the indicated wild-type or mutant Mcm2-7 preparation.

Mcm2-7 into functional Mcm467 complexes. Immunoprecipitation of Mcm2-7 with a Mcm4-specific antibody in the presence of either 200 mM potassium chloride or potassium glutamate was equally efficient and yielded identical results (Figure S5A), arguing against the bulk disruption of Mcm2-7 by glutamate. The possibility remains, however, that glutamate liberates a small but catalytically significant amount of Mcm467.

To test this possibility directly, we examined our collection of Mcm antibodies for any that would neutralize only one of the two complexes. Two antibodies were identified that specifically inhibit Mcm467 helicase activity with no effect upon Mcm2-7 activity: one targets the extreme N-terminus of Mcm7 (SC-7) while the other targets the Walker B motif common to all six subunits (AS4.1) (Figure 4E, compare lanes 5 and 6 with 10 and 13). The reversal of helicase inhibition by specific blocking peptides confirms the antibody specificity (compare lanes 10 to 11 and 13 to 14). Although neither antibody blocks the binding of Mcm467 to ssDNA (data not shown), SC-7 is able to dissociate the Mcm467 hexamer, but not Mcm2-7 (Figure S5B), suggesting a possible mechanism for Mcm467 inactivation. These data strongly indicate that the helicase activity of Mcm2-7 does not involve the fortuitous generation of Mcm467.

To verify the subunit composition of the active helicase, Mcm2-7 complexes containing Walker A mutations in individual subunits were assayed. Although Walker A mutations in Mcm4, Mcm6, and Mcm7 completely abolish helicase activity (Figure 4F, lanes 6, 8, and 9), analogous mutations in Mcm2 and Mcm5 eliminate (Mcm5) or considerably reduce (Mcm2) helicase activity (Figure 4F, lanes 4 and 7). In addition, the complex containing the 2RA mutation is completely devoid of activity, confirming the involvement of Mcm2 in helicase activity (Figure 4F, lane 14). In contrast, the Mcm3KA complex retains nearly wild-type levels of helicase activity, suggesting that this ATP binding site may not be essential for helicase activity.

Although Walker A mutations in individual Mcm subunits block ATPase activity of Mcm2-7, double or triple mutant complexes that contain combinations of the Mcm2KA, -3KA, or -5KA subunits restore ATPase activity (Schwacha and Bell, 2001). To test if these combinations also restore helicase activity, we assayed Mcm2-7 complexes containing two (2/3KA, 3/5KA, or 2/5KA) or three (2/3/5KA) mutant subunits (Figure 4F, lanes 10–13). The recovery of ATPase activity in these mutants does not result in a reactivation of helicase activity. In combination, our results confirm the involvement of at least five of the six subunits in Mcm2-7 helicase activity.

DISCUSSION

The prior observation that Mcm2-7 lacks helicase activity can now be rationalized. The anions present in previously published

helicase assays would have blocked Mcm2-7-unwinding activity (Ishimi, 1997; Kaplan et al., 2003; Lee et al., 2000). The correlation between inability to bind circular ssDNA and helicase activity suggests that circularization of the complex is essential for DNA unwinding, an observation supported by studies of other hexameric helicases (e.g., Schuck and Stenlund, 2005).

Our findings appear to conflict with a recent study indicating that Cdc45 and the GINS complex are required for in vitro Mcm2-7 helicase activity (CMG complex, Moyer et al., 2006). Although our data clearly demonstrate that Ccd45 and the GINS complex are not absolutely required for in vitro Mcm2-7 helicase activity, our results do not preclude the possibility that these factors may assist Mcm2-7 unwinding activity under more stringent in vitro or in vivo conditions. Indeed, our findings suggest that Ccd45 or the GINS complex may act on the Mcm2-7 complex to close the Mcm2-7 ring. As has been true for elucidating the mechanism of DNA replication in *E. coli*, the function of individual components is frequently obtained by studying both whole complexes and their parts. For example, it was the study of the gamma complex, not the DNA pol III holenzyme, that identified its clamp loading function (reviewed in Ellison and Stillman, 2001). Our studies lay the foundations to examine the mechanistic contribution of the different Mcm subunits within the Mcm2-7 helicase in a manner that will complement studies of the larger CMG complex.

Role of Glutamate and the Mcm2/5 "Gate" in Mcm2-7 Helicase Activity

Given that chloride competes with glutamate in facilitating Mcm2-7 helicase activity (Figure 3C), glutamate likely serves to displace inhibitory anions. Since DNA is itself negatively charged, helicase stimulation cannot involve the binding of glutamate to DNA. Moreover, since glutamate changes the ability of Mcm2-7 to bind different topological forms of ssDNA rather than changing its affinity for ssDNA (Figure 4A), glutamate likely facilitates a conformational change in the complex.

The finding that glutamate mimics many of the effects of ATP, a known Mcm2-7 substrate, further suggests the physiological relevance of the effects of glutamate. Although chloride is commonly present in many in vitro enzymatic reactions, it is not the major cellular anion in eukaryotes (see Leirmo et al., 1987 and references therein).

The inverse correlation between circular ssDNA binding and helicase activation lead us to hypothesize that this conformational change corresponds to topological closure of the Mcm2-7 toroid. Consistent with this notion, the minus 6 pentamer, which is presumably unable to fully circularize, lacks helicase activity (data not shown). Analysis of the Mcm5KA and Mcm2RA mutant complexes suggests that the Mcm2-7 discontinuity corresponds to the Mcm2/5 ATPase active site. Interestingly, these two mutations have opposite effects on gate closure: Mcm5KA increases the ability of Mcm2-7 to bind circular ssDNA (i.e., gate open), whereas the 2RA mutation decreases its ability to bind circular ssDNA (i.e., gate closed). Studies of other AAA^+ proteins show that mutations in the Walker A motif commonly block both ATP binding and hydrolysis, whereas arginine finger mutants likely bind ATP but are unable to hydrolyze it (Hanson and Whiteheart, 2005). If this precedence applies to Mcm2-7, it suggests that ATP (ATPγS) binding, but not hydrolysis, stimulates closure of the gate. Since the Mcm2RA complex lacks helicase activity (Figure 4F, lane 14), we infer that at some stage during DNA unwinding, ATP hydrolysis at the Mcm2/5 site is required. Although our data suggest that the Mcm2/5 ATPase active site is the gate, we cannot preclude the possibility that additional residues involved in the dimer interface (such as the N-terminus [Fletcher et al., 2003]) also contribute to gate formation. A previous study indicating that the Mcm2/5 active site is particularly labile (Davey et al., 2003) further supports our proposal. We note, however, that since ATP preincubation by itself is unable to stimulate Mcm2-7 helicase activity, glutamate must serve an additional role in helicase activation, perhaps to displace inhibitory ions from additional sites in Mcm2-7.

Possible Role of the Mcm2/5 Gate during DNA Replication

An ATP-regulated gate in Mcm2-7 has obvious potential implications for the loading or activation of the complex during DNA replication (Figure S6). Since Mcm2-7 oligomerizes prior to nuclear transport (reviewed in Bell and Dutta, 2002), it is likely that toroid opening is needed to allow DNA passage into the central channel. The relative weakness of the Mcm2/5 active site makes this an ideal entry point for DNA, suggesting that ATP hydrolysis at the Mcm2/5 site may facilitate Mcm2-7 loading onto replication origins. In addition, our results suggest that the in vitro helicase activity of Mcm2-7 requires closure of the Mcm2/5 gate. In vivo, the helicase activity of the Mcm2-7 complex appears to be blocked until the G1/S transition (Geraghty et al., 2000). Origin unwinding depends on the activity of the Cdc7/Dbf4 kinase (Geraghty et al., 2000), and the Mcm2-7 complex is widely believed to be the main substrate for this kinase. Although in vivo the relatively high cellular levels of acetate or glutamate may favor transient Mcm gate closure, Cdc7/Dbf4 phosphorylation and the subsequent association of Cdc45 and GINS may serve to stabilize this closure to enable Mcm2-7 to processively unwind large regions of chromatin.

EXPERIMENTAL PROCEDURES

Proteins and Purification

Proteins were prepared as described (Bochman and Schwacha, 2007; Schwacha and Bell, 2001), and concentrations are expressed as hexamers (or pentamers for the minus 6 prep). Mcm preparations were analyzed for subunit composition using quantitative westerns and hexameric size by gel filtration (Figures S1B–S1E). We estimate that $\geq$ 50% of the Mcm subunits present in our wild-type Mcm2-7 and Mcm467 preparations and $\geq$ 30% in our various mutant preparations are ssDNA binding competent hexamers (see legend for Figure S1).

Antibodies

Antibodies are from Santa Cruz Biotechnology (anti-Mcm2 [sc-6680] and SC-7 [sc-6688]) or are mouse monoclonals (anti-Mcm4 [AS6.1]) and two different antibodies that recognize the Walker B box on all 6 subunits, (AS1.1 and AS4.1) (Bochman and Schwacha, 2007; A.S. and S. Bell. unpublished data). Blocking peptide (NP-2) for SC-7 (sc-6688P) was from Santa Cruz Biotechnology, and blocking peptides to AS4.1 and AS1.1 (Walker B sequence, IDEFDKMAD, neutralizing peptide 1, NP-1) and a randomized Walker B sequence (IEKFMDDAD, randomized peptide, RP) were prepared by the Peptide Synthesis Facility at the University of Pittsburgh.

Double Filter-Binding Assay

The Mcm/ssDNA filter-binding assay has been described (Bochman and Schwacha, 2007). Oligonucleotide substrates were radiolabeled using T4 polynucleotide kinase and γ^{32}P-ATP. Several different oligonucleotides are used as indicated since at least one (oligo no. 455) gives high nonspecific background in the presence of glutamate; however, in all cases when results are being compared, identical oligos are used. Variations on the standard reaction are described in the Supplemental Experimental Procedures. To remove contaminating M13 linear ssDNA from our circular ssDNA preparations, 75 units of T4 DNA polymerase were added per microgram of ssM13 DNA in NEB buffer 2 without dNTPs for 1 hour at 37°C (Figure S1A). Linear M13 ssDNA was generated by digestion of the circular M13 ssDNA with HhaI; HhaI cleaves ssDNA at its usual consensus sequence (Nishigaki et al., 1985), generating approximately 22 ssDNA fragments between 965 and 20 nucleotides in length (Figure S1A). Data represent the averages of $\geq$ 3 repeats; error bars correspond to the standard deviation. Binding constants were calculated as before (Bochman and Schwacha, 2007), and IC_{50} values were derived from fitting the data to an exponential decay curve using SigmaPlot.

Helicase Assay

The helicase assay was based on (Kaplan et al., 2003) and modified as described (Bochman and Schwacha, 2007). Synthetic replication forks were prepared by annealing oligos nos. 233 and 235 (Table S1) and then filling them in with α-^{32}PdATP/cold dNTPs using reverse transcriptase. The figure legends describe variations of the standard reaction. For antibody neutralization of the Mcm467 helicase, a 1:6 dilution of the mouse monoclonal or a 1:30 dilution of the Santa Cruz antibodies were added to Mcm proteins and incubated for 30 min at 30°C prior to addition of DNA and ATP. Blocking peptides were added to a final concentration of $\leq$0.8 ng/μl and incubated with the antibodies for 30 min at 30°C prior to Mcm addition.

Nucleotides, DNA, Buffers, and Other Reagents

Radiolabeled nucleotides were from Perkin Elmer or MP Biomedical, unlabeled ATP was from GE Healthcare, and oligonucleotides were from Integrated DNA Technologies (Coralville, IA) (Table S1). Circular single- and double-stranded M13mp18 DNA were from Bayou Biolabs (Harahan, LA). Nucleotide and DNA concentrations were calculated from 260 nm absorbance. Buffers B2 (Bochman and Schwacha, 2007) and helicase buffer (Kaplan et al., 2003) are as previously described.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, one table, and six fugures and can be found with this article online at http://www.molecule.org/cgi/content/full/31/2/287/DC1/.

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