Roles of Mcm7 and Mcm4 Subunits in the DNA Helicase Activity of the Mouse Mcm4/6/7 Complex*

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Mcm, which is composed of six structurally related subunits (Mcm2–7), is essential for eukaryotic DNA replication. A subassembly of Mcm, the Mcm4/6/7 double-trimeric complex, possesses DNA helicase activity, and it has been proposed that Mcm may function as a replicative helicase at replication forks. We show here that conserved ATPase motifs of Mcm7 are essential for ATPase and DNA helicase activities of the Mcm4/6/7 complex. Because uncomplexed Mcm7 displayed neither ATPase nor DNA helicase activity, Mcm7 contributes to the DNA helicase activity of the Mcm complex through interaction with other subunits. In contrast, the Mcm4/6/7 complex containing a zinc finger mutant of Mcm4 with partially impaired DNA binding activity exhibited elevated DNA helicase activity. The Mcm4/6/7 complex containing this Mcm4 mutant tended to dissociate into trimers, suggesting that the zinc finger of Mcm4 is involved in subunit interactions of trimers. The Mcm4 mutants lacking the N-terminal 35 or 112 amino acids could form hexameric Mcm4/6/7 complexes, but displayed very little DNA helicase activity. In conjunction with the previously reported essential role of Mcm6 in ATP binding (You, Z., Komamura, Y., and Ishimi, Y. (1999) Mol. Cell. Biol. 19, 8003–8015), our data indicate distinct roles of Mcm4, Mcm6, and Mcm7 subunits in activation of the DNA helicase activity of the Mcm4/6/7 complex.

DNA helicase plays a central role in the replication of eukaryotic DNA. It couples the free energy of ATP hydrolysis to the separation of a DNA duplex into its component strands. DNA helicase is associated with a set of subactivities including DNA binding, nucleotide binding, and ATP hydrolysis, all of which cooperatively work for DNA unwinding (1, 2). The Mcm family proteins (Mcm2–7) interact with one another to form a complex that plays an essential role in the initiation of DNA replication (3–7). A recent demonstration that the Mcm4/6/7 complex can form a hexameric complex with active helicase activity suggests that the Mcm complex may be part of the replicative DNA helicase of eukaryotes (8–13).

All six Mcm proteins contain DNA-dependent ATPase motifs in their central domains, including Walker motifs A and B, which are widely conserved in ATPases and DNA helicases (14, 15). In addition, four of the Mcm proteins (Mcm2, Mcm4, Mcm6, and Mcm7) contain a zinc finger motif, which may function in protein-protein interactions or nucleic acid binding (3, 16). The ATPase and zinc finger motifs are highly conserved in Mcm proteins from yeast to mammalian cells, suggesting their crucial roles in the functions of Mcm.

We have identified DNA helicase activity in the human Mcm4/6/7 protein complex (8) and subsequently demonstrated that it is intrinsic to mammalian Mcm4/6/7 complexes (9). DNA helicase activity was also demonstrated in the Mcm4/6/7 complex of fission yeast (10, 11). To elucidate the mechanism of the DNA helicase functions of the Mcm4/6/7 complex and to obtain insight into the physiological significance of this activity, biochemical studies with a series of mutants of the Mcm4/6/7 complex were undertaken. Our previous studies with mutant Mcm4/6/7 complexes in which the ATP-binding motifs of the Mcm6 protein were mutated showed that the mutation preferentially affected the high affinity binding of ATP and inactivated the DNA helicase activity, although the ATPase activity was not significantly affected. A mutation in an ATP-binding motif of the Mcm4 protein affected the ssDNA binding activity of the complex and led to moderate inhibition of the DNA helicase activity (9). In the present study, the roles of ATP-binding motifs of Mcm7 and those of the zinc finger motif of Mcm4 in various activities of the Mcm4/6/7 complex were examined. Our results indicate that ATPase motifs of Mcm7 are essential for the ATP hydrolytic and DNA helicase activities of the Mcm4/6/7 complex, whereas Mcm7 alone does not possess these activities, and that zinc finger motif mutants of Mcm4 display enhanced DNA helicase activity, with altered subunit assembly and partially impaired DNA binding activity. We have shown that the N-terminal region of the Mcm4 protein is also required for DNA helicase activity. Our results indicate distinct roles of each subunit of the Mcm4/6/7 complex in the execution of its helicase activity. A possible model of the helicase action of Mcm is discussed on the basis of these findings.

EXPERIMENTAL PROCEDURES

Generation of Mutant Forms of Mouse Mcm7 and Mcm4 cDNAs—Site-directed mutagenesis of the Mcm7 and Mcm4 genes was conducted with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotides 5′-GGGTGTGCTGATTGCTTTCAGTACAAGATGCCC-3′ and 5′-GACCTTGTGTCCCAGCAGTCAG-3′ were used as primers.

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CTCCTATCTTAC-3' were used as primers to prepare the histidine-tagged Mcm7 D10E and Mcm7 K80R mutants (where "DE" refers to the Asp^34 and Glu^44 residues in the Walker B motif mutated to alanine, and "KS" refers to the highly conserved Lys^285 and Ser^287 residues in the Walker A motif mutated to alanine), respectively. Mcm7 D10E and Mcm7 K80R were used as primers to introduce Cys-to-Ala mutations at amino acids 305 and 308 amino acids 237 and 330 of Mcm4 in pBluescript II SK− (−), resulting in Mcm4 C4- and Mcm4 C4, respectively. Deletion mutants of Mcm4 were generated by PCR amplification first, to amplify the N-terminal fragments of the Mcm4 gene (amino acids 35–148 and 114–148 as EcoRI-HindIII fragments, respectively, in combination with 5'-TGTTTTCCCAGTCACGAC-3' and 5'-GCTGAGGCTCTGATGCTGTGACAGCGGAC-ACATCATCACGGAAGACTAGAGGCGAAGATTC-3' oligonucleotides 5'-GGAGAGAGAATTCATGGGACATCATAC-3' or 5'-GAGAGAGAATTCATGGGACATCATCATCACTACCAAGGAGCTGAGGCAAGATGCT-3', respectively). The amplified fragments were digested with EcoRI plus HindIII and ligated to the Mcm4 C-terminal fragment (amino acids 149–862), resulting in Mcm4 C4- and Mcm4 C4- (Stratagene). Mcm4 mutants were synthesized on these templates using DNA in vitro in the presence of [35S]methionine in the Tnt-coupled reticulocyte lysate system (Promega, Madison, WI) as suggested by the manufacturer. The reaction mixture was diluted with 400 μl of buffer B (20 mM Tris-HCl (pH 8.0), 0.3 mM sodium glutamate, 2 mM Mg(CH3COO)2, 10% glycerol, 0.01% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) and then concentrated to 50 μl using a Microcon-10 column (Amicon, Inc.). This procedure removed free [35S]methionine from the reaction mixtures. The TNT product of Mcm4 (wild-type or mutant) was mixed with an equal amount of Mcm6 or Mcm7, diluted to 30 μl with buffer B containing 1 mM ATP, and then incubated with 1 μl of anti-mouse Mcm4 antibody (serum generously provided by Dr. Hiroshi Hirotani, RIKEN, Japan) at 4°C for 2 h. Protein A-Sepharose (15 μl) and the anti-Mcm4 antibody were added, and the incubation was continued for an additional 1 h. Immuno complexes were washed five times with buffer B containing 1 mM ATP; the bound and unbound proteins were electrophoresed on 8% SDS-polyacrylamide gel, and the radioactivity on the gel was analyzed with the Fuji Bio-Image analyzer.

RESULTS

Expression and Purification of Mouse Mutant Mcm4/6/7 Complexes Containing ATPase Motif Mutations in Mcm7—Two mutations that changed specific amino acids located in the well conserved DNA-dependent ATPase motifs of Mcm7 were generated by site-directed mutagenesis (Fig. 1). The highly conserved lysine and serine residues (KS) in the Walker A motif and the aspartic and glutamic acid residues (DE) in the Walker B motif were changed to alanine in the Mcm7 protein (Mcm7 KS and Mcm7 DE, respectively). These amino acids have been implicated in the nucleotide hydrolytic and DNA unwinding activities of various ATPases and DNA helicases (18–22). To express these mutant Mcm4/6/7 proteins, HighFive insect cells were co-infected with two recombinant baculoviruses, Mcm2/His6-Mcm7 (DE or KS) and His6-Mcm4/6/7. The mutant Mcm4/6/7 complexes were purified to near homogeneity by Ni^2+ -agarose chromatography, histone- Sepharose column chromatography, and glycerol gradient centrifugation (9) as described previously (Fig. 2A). Glycerol gradient sedimentation indicated that the mutant complexes sedimented at 550 kDa, a value almost identical to that of the wild-type complex (data not shown). Examination of the purified proteins by SDS-PAGE revealed that the three subunits largely formed stoichiometric complexes in both mutants (Fig. 2A). The levels of contaminating Mcm2 protein in the purified Mcm4/6/7 complexes were examined by Western blotting (Fig. 2B). The results show that the amounts of Mcm2 protein present in the Mcm4/6/7 complexes were similar in the wild-type and mutant Mcm7 proteins and were <5% of the ssDNA Binding and ATP Binding Assays—Mcm proteins were incubated with the labeled 37-mer oligonucleotide (9) at 37°C in buffer containing 10 mM sodium creatine phosphate, 5 mM ATP, 5 mM MgCl2, 0.3 mM DTT, 0.01% Triton X-105, and 15 mM potassium phosphate (pH 7.5) for 30 min. Glutaraldehyde (10%) was then added to the reaction oligonucleotide was added. After incubation at 37°C for 1 h, aliquots (0.5 μl) were spotted onto a polyethyleneimine-cellulose TLC plate, and the ATP and P were separated by chromatography in 1 M formic acid and 0.5 M LiCl. The extent of ATP hydrolysis was quantified by using a Fuji BAS 2500 Bio-Image analyzer.
amount present in the helicase-inactive Mcm2/4/6/7 complex containing the same amounts of Mcm4, Mcm6, and Mcm7 subunits (Fig. 2B, "upper panel") (data not shown). This level of Mcm2 does not inhibit the helicase activity of the Mcm4/6/7 complex (9). Analyses of the mutant complexes on a nondenaturating acrylamide gel indicated that they predominantly existed as hexameric complexes, like the wild-type complex (Fig. 2C), indicating that complex formation is not affected by the Mcm7 mutations.

**Mutations in the Walker A or B Motif of the Mcm7 Protein Result in Reduction of ATPase and DNA Helicase Activities**—We then characterized the biochemical properties of the mutant complexes. The mutations resulted in almost no (Mcm4/6/7X1) or significantly reduced (Mcm4/6/7X2) DNA helicase activity (Fig. 3A). The levels of ATPase activities of the mutant complexes were also significantly lower than that of the wild-type Mcm4/6/7 complex (Fig. 3B). However, they retained ssDNA and ATP binding activities that were nearly comparable to those detected with the wild-type complex (Fig. 3, C and D). The above results indicate that ATPase motifs of Mcm7 are essential for the DNA helicase and ATP hydrolytic activities expressed by the Mcm4/6/7 complex.

**Uncomplexed Mcm7 Protein Does Not Have DNA Helicase or ATPase Activity**—The above results suggest that Mcm7 may directly contribute to the DNA helicase and ATPase activities of the Mcm4/6/7 complex. Therefore, we examined whether a single polypeptide of the Mcm7 protein possesses ATP hydrolytic and DNA helicase activities. The Mcm7 protein fraction obtained after three steps of purification (see "Experimental Procedures") was further fractionated by glycerol gradient centrifugation. The proteins in these fractions were then analyzed by SDS-PAGE (Fig. 4A). The major peak of the Mcm7 protein sedimented at fractions close to catalase (230 kDa), indicating that Mcm7 may form a trimeric complex. The ATPase and DNA helicase activities were then measured using this purified protein fraction. As shown in Fig. 4 (B and C), neither ATPase nor DNA helicase activity was detected in the Mcm7 protein preparations. These results indicate that Mcm7 itself is deficient in ATP hydrolytic and DNA helicase activities and suggest that it may contribute to the DNA helicase and ATPase activities of the Mcm4/6/7 complex through interacting with other subunits.

**The Mcm4/6/7 Complex with a Mutation in the Zinc Finger Motif of Mcm4 Tends to Disassemble into Trimers**—Four of the six Mcm subunits (Mcm2, Mcm4, Mcm6, and Mcm7) contain a conserved zinc finger motif (CXXC/CX3CX2C), suggesting that they may be involved in protein-protein interactions, protein folding stability, or DNA-protein interactions (24–26). To elucidate the roles of the zinc finger motif of the Mcm4 protein in the biochemical activities of the Mcm4/6/7 complex, we replaced the first or second pair of cysteines in Mcm4 with alamine by site-directed mutagenesis and expressed it as an Mcm4/6/7 complex (Mcm4CS1/6/7 or Mcm4CS2/6/7, respectively) (Fig. 1). These mutant complexes, carrying a histidine tag and a FLAG tag at the N terminus of Mcm4 and at the C terminus of Mcm7, respectively, were purified by Ni2+-nitrilotriacetic acid affinity chromatography.
acid column chromatography, FLAG affinity column chromatography, and glycerol gradient centrifugation (Fig. 5A). The wild-type Mcm4/6/7 complex sedimented at 500–600 kDa, whereas the Mcm4 CC2/6/7 complex peaked at fractions slightly larger than catalase (232 kDa) (Fig. 5A, upper panels). Examination of each fraction on a nondenaturing acrylamide gel revealed that the mutant complex contained a significant amount of 280-kDa trimers, whereas the wild-type complex was almost exclusively composed of 550-kDa hexameric complexes (Fig. 5A, lower panels). Similar results were obtained with the Mcm4CC1/6/7 complex (data not shown). These results indicate that mutations in the zinc finger result in disassembly of the hexameric complex into trimers. Therefore, the zinc finger motif of Mcm4 is likely to be involved in the stable assembly of the hexameric structure.

To address the molecular basis of the instability of the Mcm4/6/7 hexamer containing an Mcm4 zinc finger mutant, we examined the interaction of wild-type or mutant Mcm4 with Mcm6 and Mcm7. When in vitro synthesized wild-type Mcm4 was mixed with Mcm6 or Mcm7 similarly synthesized in vitro, anti-Mcm4 antibody efficiently immunoprecipitated Mcm6 or Mcm7, respectively (Fig. 5B, lane 2). Approximately 20% of the input Mcm6 and Mcm7 was immunoprecipitated. Lower bands in the bound fraction may be degradation products of Mcm4. These results indicate that Mcm4 interacts with both Mcm6 and Mcm7 under our experimental conditions. The Mcm6 and Mcm7 proteins were co-immunoprecipitated with the mutant Mcm4 proteins with similar efficiency (Fig. 5B, lanes 3 and 4), indicating that the zinc finger mutations of Mcm4 do not affect its interaction with the Mcm6 or Mcm7 protein. We speculate that they may alter the overall structure of the Mcm4/6/7 protein, thus affecting the dimerization of the Mcm4/6/7 trimeric complex.

Increased DNA Helicase Activity of Mcm4/6/7 with Mcm4 Zinc Finger Mutants—The purified Mcm4/6/7 complexes containing the zinc finger mutants contained the three subunits at a stoichiometry identical to that of the wild-type complex, as judged by SDS-PAGE analyses (Fig. 5C). The two zinc finger mutant Mcm4/6/7 complexes retained the DNA-dependent ATPase activities at a level comparable to that of the wild-type complex in the presence of ssDNA (Fig. 5D) (data not shown).

In gel shift assays, the complex formation was significantly reduced in the Mcm4CC1/6/7 complex and reduced by ~50% in Mcm4CC2/6/7 (Fig. 5E). A similar reduction of DNA binding was...
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Fig. 4. Purification of uncomplexed Mcm7 protein and its ATPase and DNA helicase activities. The Mcm7 protein fraction, prepared by anti-FLAG antibody affinity column chromatography, was fractionated by centrifugation at 36,000 rpm for 16 h on a 15–35% glycerol gradient, and each fraction was subjected to 10% SDS-PAGE, followed by silver staining (A). The positions of molecular mass markers (Thy, thyroglobulin; Cat, catalase; BSA, bovine serum albumin) are indicated along with their molecular sizes. Fractions 8 and 9 on the glycerol gradient were pooled and concentrated, and ATPase (B) and DNA helicase (C) activities were examined as described under “Experimental Procedures.” In B and C, the released phosphate (P, in pmol; the background in the absence of added protein was taken as 0) and displaced oligonucleotides (% of the total) were quantified, and the values are presented at the bottom of each lane.

observed also in filter binding assays with the mutant complexes (Fig. 5F). These results indicate that the zinc finger mutations reduce the DNA binding activity of the Mcm4/6/7 complex.

In contrast, these mutant complexes exhibited increased DNA helicase activities. The specific activities of both zinc finger mutants were 2-fold higher than that of the wild-type complex (Fig. 5G). Possible mechanisms of how reduced stability of hexameric structures and DNA binding activity may contribute to enhanced DNA helicase activity will be discussed later.

Attenuation of DNA Helicase Activity in Mcm4/6/7 Containing an N-terminal Deletion of Mcm4—The N-terminal region of Mcm4 contains arginine clusters and several potential cyclin-dependent kinase phosphorylation sites (T/S)XXP or (S/T)P) (Fig. 6A). It has been suggested that the phosphorylation of Mcm4 is involved in the regulation of chromatin binding of Mcm proteins (27) or in the inhibition of the DNA helicase activity of the Mcm4/6/7 complex (28, 29). Serine/arginine residues have been implicated in nucleic acid binding (30, 31). N-terminal 35- and 112-amino acid deletions were introduced into histidine-tagged Mcm4, and the resulting truncated proteins (His6-Mcm4ΔN) were coexpressed in insect cells with Mcm6 and FLAG-tagged Mcm7. Mcm4ΔN,6/7 and Mcm4ΔN,112/7 were purified by a procedure similar to that used for the other mutants. The truncated Mcm4 polypeptides contained in the purified complexes migrated upon SDS-PAGE as expected (Fig. 6B). The N-terminal deletion of Mcm4 did not significantly affect the assembly of the mutant Mcm4 proteins into the Mcm4/6/7 hexameric complex (Fig. 6B) (data not shown). The two truncated Mcm4 mutants were poorly phosphorylated by Cdk2/cyclin A kinase in vitro, consistent with the notion that the major cyclin-dependent kinase phosphorylation sites of Mcm4 lie in the N-terminal region of the protein (28, 29) (data not shown).

These two mutants displayed significantly reduced DNA helicase activity (Fig. 6C). In contrast, the ATPase and ATP binding activities of the Mcm4/6/7 complex were not affected by the N-terminal truncations of Mcm4 (Fig. 6D) (data not shown). These results suggest that the N-terminal region of Mcm4 contributes to the DNA helicase activity of the Mcm4/6/7 complex in some unknown manner.

DISCUSSION

Accumulating evidence points to an essential role of the Mcm complex as a replicative DNA helicase at replication forks. The DNA helicase activity of the Mcm4/6/7 complex, first reported in humans, now appears to be conserved generally in eukaryotes (8, 10). A previous mutational study of Mcm subunits at ATP-binding motifs indicated essential roles of Mcm4 and Mcm6 in ssDNA binding and ATP binding, respectively (9). In this study, to further elucidate the mechanisms of the DNA helicase actions of Mcm4/6/7, we analyzed the roles of the ATP-binding motifs of Mcm7 as well as those of the zinc finger motif and another domain of Mcm4 in various biochemical activities of the Mcm4/6/7 complex.

ATPase Mutants of Mcm7—We have shown here that the conserved ATP-binding motifs of Mcm7 play crucial roles in expression of the DNA helicase and ATP hydrolytic activities of the Mcm4/6/7 complex by examining the activities of an Mcm4/6/7 mutant containing an Mcm7 Walker A or B motif mutant. Mutagenesis studies with various helicases and other nucleotide-binding proteins have shown that the Walker A and B motifs are critical for nucleotide hydrolysis (18–22). In contrast, two mutant complexes showed the wild-type level of ATP and DNA binding activities (Table I), consistent with our previous results that they are mediated mainly by the Mcm6 and Mcm4 proteins, respectively (9). These results indicate that the well conserved ATP-binding motifs of Mcm4, Mcm6, and Mcm7 are required for DNA helicase functions and that each plays distinct functions in helicase activation. Because Mcm7 alone does not show any DNA helicase or ATPase activity, it contributes to the ATPase and helicase actions of the Mcm4/6/7 complex through interactions with the Mcm4 and Mcm6 proteins.

It was recently reported that the Walker motifs of all six subunits are required for nucleotide hydrolytic activity of the purified Mcm2/3/4/5/6/7 heterohexamer of Saccharomyces cerevisiae (32). Interestingly, a combination of the Walker motifs of some of the Mcm subunits reactivated the nucleotide hydrolytic activity of the complex. Thus, proper alignment of active ATPase subunits in the hexamer appears to be required for ATP hydrolytic activity. The single Walker mutation of Mcm4 or Mcm6 may be tolerated for expression of the ATPase activity of the Mcm4/6/7 complex, presumably because sequential ATP hydrolysis by component subunits can be maintained. However, it should be noted that ATP hydrolysis by the Mcm4/6/7 complex is stimulated by DNA binding, whereas that by the
Fig. 5. Mutations in the zinc finger motif of the Mcm4 protein in the Mcm4/6/7 complex result in unstable hexamers, reduced ssDNA binding activity, and increased DNA helicase activity. A, the wild-type (WT) Mcm4/6/7 (left panels) and Mcm4CC2/6/7 (right panels) complexes, purified on nickel and anti-FLAG antibody affinity columns, were separated by 15–30% glycerol gradient centrifugation as described under “Experimental Procedures.” Each fraction was loaded onto 10% SDS-polyacrylamide gel (37.5:1 acrylamide/bisacrylamide; upper left panel), 8% SDS-polyacrylamide gel (59:1 acrylamide/bisacrylamide; upper right panel), or 5% native polyacrylamide gel (lower panels); and proteins were stained with silver. B, the wild-type (WT) and mutant Mcm4, Mcm6, and Mcm7 proteins were synthesized in vitro in the presence of [35S]methionine. Approximately the same amounts of Mcm4 and Mcm6 (upper panels) or Mcm4 and Mcm7 (lower panels) were mixed as indicated, and immunoprecipitation was carried out with anti-Mcm4 antibody. Input (15% of total) and bound proteins were run on 8% SDS-polyacrylamide gel (37.5:1 acrylamide/bisacrylamide), and proteins were detected by autoradiography. C, the final preparations of the wild-type and zinc finger mutants of Mcm4/6/7 (150 ng each), used in the various assays described for D–G, were prepared by concentrating glycerol gradient peak fractions (wild-type complex, pool of fractions 3–5; and zinc finger mutant complex, pool of fractions 6–8) and subjected to 10% SDS-PAGE, followed by silver staining. D, ATPase assays were conducted under the standard conditions with various amounts of a zinc finger mutant (Mcm4CC2/6/7) or the wild-type Mcm4/6/7 complex as indicated. The reactions contained a 67-mer oligonucleotide DNA at 50 ng/µl. The released phosphate (Pi in pmol; the background in the absence of added protein was taken as 0) was quantified, and the values are presented at the bottom of each lane. E, the ssDNA binding activities of the wild-type and zinc finger mutant Mcm4/6/7 complexes were measured as described under “Experimental Procedures.” The gray arrow indicates the position of the Mcm4/6/7 hexamer (550 kDa) in complex with the 37-mer DNA. The positions of protein molecular mass markers (Thy, thyroglobulin; Fer, ferritin) upon glycerol gradient and native gel electrophoresis are indicated by black arrows. The protein-free oligonucleotides have run off the gel. The intensities of the shifted bands were quantified, and the relative extent of binding, with the intensity of the shifted DNA with 50 ng of the wild-type protein being taken as 100, is presented below each lane. F, nitrocellulose filter binding assays of the wild-type and mutant Mcm6/6/7 complexes were conducted with 20 fmol of labeled 37-mer oligonucleotide. The amounts of DNA-protein complexes generated are plotted against the concentration of Mcm proteins. The values are the averages of three independent experiments, and error bars are shown. G, the DNA helicase activities of the zinc finger mutant Mcm4/6/7 complexes, as indicated, were measured along with the wild-type complex as described under “Experimental Procedures.” The displaced oligonucleotides (%) were quantified and are plotted.
Mcm2/3/4/5/6/7 hexameric complex is independent of DNA (32). Thus, the mechanisms of ATP hydrolysis by both complexes may be intrinsically different from each other. It was shown that T7 phage-encoded DNA helicase (T7gp4) in hexamer form possesses two classes of dTTP-binding sites and catalyzes the sequential hydrolysis of two nucleotides (33). The helicase action of Mcm4/6/7 may be similar to that of T7gp4 in that two Mcm7 molecules in the Mcm4/6/7 hexamer could be responsible for sequential ATP hydrolysis, and the “two-site sequential NTPase model” (1) may apply for Mcm4/6/7 helicase.

Mutations in Mcm4 Affect DNA Binding and Helicase Activities—On the other hand, zinc finger mutations of Mcm4 showed partially impaired ssDNA binding activity and a tendency to disassemble into trimers. These mutants exhibited 2-fold higher DNA helicase activity compared with the wild-type protein. It was recently reported that an archaeal Mcm mutant containing a similar zinc finger mutation showed no DNA helicase and ATPase and reduced DNA binding activities (34), indicating essential roles of the zinc finger structures in its helicase function. Archaeal Mcm is a homohexameric helicase, whereas mammalian Mcm4/6/7 is a dimer of heterotrimers. Construction and characterization of similar zinc finger mutants of Mcm6 and Mcm7 will provide further clues to the precise roles of zinc fingers of the mammalian Mcm complex. In contrast, the DNA helicase activity was severely impaired in the Mcm4/6/7 complex containing an N-terminally truncated Mcm4 protein. We previously reported that a Walker A mutant of Mcm4 led to reduced ssDNA binding and DNA helicase activities (9). These results indicate essential roles of the Mcm4 protein in the helicase actions of Mcm4/6/7, presumably by affecting its DNA binding activity.

Possible Mechanism of Stimulation of DNA Helicase Activity in Mcm4 Zinc Finger Mutants—Zinc finger motifs play important roles in protein-protein and DNA-protein interactions (24–26). Our results indicate that mutations in the zinc finger motif of the Mcm4 protein destabilize the hexameric structures, which are the functional forms for binding to ssDNA. This may be the reason why these mutants show reduced binding to ssDNA. The zinc finger regions of SV40 and polyoma virus T antigens were also reported to contribute to protein-protein interactions required for hexamer and double-hexamer formation at the origins (35, 36). Co-immunoprecipitation assays did not show a defect in the interaction of the Mcm4 mutant with Mcm6 or Mcm7, and the mutant Mcm4/6/7 trimeric complexes with stoichiometric composition could be purified from baculovirus-infected HighFive cells on affinity columns. Thus, the zinc finger motif of Mcm4 is likely to be involved in the assembly of the Mcm4/6/7 trimeric complex into hexamers, and the assembly reaction requires self-interaction of the Mcm4 protein. The mutant Mcm complexes exhibited over 2-fold higher levels of helicase activity compared with the wild-type complex. It was previously reported that the DNA helicase activity of a monomeric protein...
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FIG. 6. Biochemical properties of mutant Mcm4/6/7 complexes containing N-terminally truncated Mcm4. A, amino acid sequence of the N-terminal region of mouse Mcm4. Consensus sites for phosphorylation by cyclin-dependent kinases ((S/T)XXR(K), SP and TP sequences, and the arginine residues (R or SR) that may be involved in DNA binding are indicated by double underlining, single underlining, and boldface letters, respectively. B, mutant Mcm4/6/7 complexes (Mcm4<sub>N35</sub>/6/7 and Mcm4<sub>N112</sub>/6/7) were separated on a 15–30% glycerol gradient; each fraction was electrophoresed on 10% SDS-polyacrylamide gel (37.5:1 acrylamide/bisacrylamide); and proteins were stained with silver.

Table I

| Mutant          | DNA helicase | ATPase | ssDNA binding | ATP binding |
|-----------------|--------------|--------|---------------|------------|
| Mcm4/6/7 (wild-type) | ++++"        | ++++   | ++++          | ++++       |
| Mcm4<sub>N35</sub>/6/7 | -           | ++++   | +             | -          |
| Mcm4<sub>N112</sub>/6/7| -          | +      |               |            |
| Mcm4/6/7<sub>OE</sub> | -           | -      | ++            | +          |
| Mcm4/6/7<sub>GS</sub> | +           | -      | +             | +          |
| Mcm4<sub>N35</sub>/6/7<sub>RE</sub> | +     | +++    | ++            | +          |
| Mcm4<sub>N112</sub>/6/7<sub>GS</sub> | +     | +      | +             | +          |
| Mcm4<sub>CC1</sub>/6/7 | ++++        | ++++   | +             | ND         |
| Mcm4<sub>CC2</sub>/6/7 | ++++        | ++++   | +             | ND         |

"+++ ++++, 140–160% of the wild-type level; ++++, wild-type level of activity; ++, 60–80% of the wild-type level; +, 40–60% of the wild-type level; −, not detectable; ND, not done. The upper half is a summary of our previous study (9), and the lower half is a summary of this study.

SV40 T antigen was ~2-fold higher than that of a hexamer (37). Thus, stimulation of DNA helicase activity by destabilization of a hexamer may be common to hexameric helicases.

DNA helicases interact with DNA in a sequence-independent manner, and their binding to DNA should be flexible enough to permit their mobility along the DNA strand. Attenuated DNA binding caused by a mutation in the zinc finger region of Mcm4 may generate more mobility on DNA, leading to more active helicase. The zinc finger region may be involved in transient binding and release of DNA required for processive helicase action. Thus, the zinc finger region of Mcm4, essential for regulated helicase actions as a hexamer, may contribute to both protein-protein and DNA-protein interactions in the Mcm complex. The zinc finger domains of other Mcm subunits (Mcm2, Mcm6, and Mcm7) may also play similar roles. Mutagenesis studies in yeast indicated that the zinc finger structure of Mcm2 is crucial for its functions in vivo (38).

Role of the N-terminal Region of Mcm in DNA Helicase Activity—Deletion of the N-terminal 35 or 112 amino acids of Mcm4 resulted in almost completely impaired DNA helicase, but did not affect the ability to form hexameric complexes and to hydrolyze ATP. The effects of the N-terminal deletions of Mcm4 on the DNA binding activity of Mcm4/6/7 were complex. Mcm4<sub>N35</sub> showed reduced activity of ~40%, whereas Mcm4<sub>N112</sub> showed an ~50% increase in DNA binding (Table I) (data not shown). The deleted segment contains arginine-rich stretches of amino acids that may be involved in interaction with DNA. It was previously reported that a mutation in a similar arginine-rich segment (RXXRXXR) of the DnaB protein, the replicative helicase of bacteria, results in decreased DNA helicase and...
DNA binding activities (39). It should be noted that the N-terminal region of Mcm4 contains multiple serine/threonine residues, some of which may be phosphorylation sites of cyclin-dependent kinase and other kinases. It is an intriguing possibility that the interaction of Mcm with DNA and its helicase activity are regulated by phosphorylation of the N-terminal region of the Mcm4 protein. However, at the moment, we cannot rule out the possibility that the truncations caused alterations of the overall structure of the protein complex.

Both a Walker A mutation described previously (Table I) (9) and a zinc finger mutation described in this study resulted in reduced DNA binding activity, but showed differential effects on DNA helicase activity. This indicates that these segments are involved in interaction with DNA in distinct manners. The zinc finger mutation may alter the overall structure of the protein complex. The actions of this putative eukaryotic replicative helicase. The three heterologous subunits, may require complex and coordinated roles for each subunit to permit the highly regulated actions of Mcm more precisely.

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REFERENCES

1. Patel, S. S., and Picha, K. M. (2000) Annu. Rev. Biochem. 69, 651–697
2. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
3. Kelly, T. J., and Brown, G. W. (2000) Annu. Rev. Biochem. 69, 829–880
4. Tye, B. K. (1999) Annu. Rev. Biochem. 68, 649–686
5. Tye, B. K., and Sawyer, S. (2000) J. Biol. Chem. 275, 34833–34836
6. Diffley, J. F. (1996) Genes Dev. 10, 2819–2830
7. Lei, M., and Tye, B. K. (2001) J. Biol. Chem. 276, 1447–1454
8. Ishimi, Y. (1997) J. Biol. Chem. 272, 24508–24513
9. You, Z., Komamura, Y., and Ishimi, Y. (1999) Mol. Cell. Biol. 19, 8003–8015
10. Ishimi, Y., and Komamura-Kohno, Y. (1996) J. Biol. Chem. 271, 18571–18578
11. Lee, J. K., and Hurwitz, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 54–59
12. Labib, K., Tercero, J. A., and Diffley, J. F. (2000) Science 288, 1643–1647
13. Aparicio, O. M., Weinstein, D. M., and Bell, P. S. (1997) Cell 91, 59–69
14. Komin, E. V. (1995) Nucleic Acids Res. 23, 2541–2547
15. Gorbalenya, A. E., and Komin, E. V. (1993) Curr. Opin. Struct. Biol. 3, 419–429
16. Kearsey, S. E., and Labib, K. (1998) Biochim. Biophys. Acta 1398, 113–136
17. McIntee, K., Weinstock, G. M., and Lehman, I. R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 857–861
18. Rehruher, W. M., and Kowalczewski, S. C. (1993) J. Biol. Chem. 268, 1292–1297
19. Washington, M. T., Rosenberg, A. H., Griffin, K., Studier, F. W., and Patel, S. S. (1996) J. Biol. Chem. 271, 26825–26834
20. Patel, S. S., Hingorani, M. M., and Ng, W. M. (1994) Biochemistry 33, 7857–7868
21. Weng, Y., Czaplinski, K., and Feit, S. W. (1996) Mol. Cell. Biol. 16, 5477–5496
22. Marintcheva, B., and Weller, S. K. (2001) J. Biol. Chem. 276, 6605–6615
23. Ishimi, Y., Ichinose, S., Omori, A., Sato, K., and Kimura, H. (1996) J. Biol. Chem. 271, 24115–24122
24. Pyle, D. V., Barewater, K. R., Glaser, P., Martineau, L., Crasue, C. T., Fabian, H., Mantsch, H. H., Barzu, O., and Gilles, A. M. (1994) Biochemistry 33, 9960–9967
25. Mackay, J. P., and Crossley, M. (1998) Trends Biochem. Sci. 23, 1–4
26. Hendrickson, M., Madine, D., Dalton, S., and Gautier, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1223–1228
27. Ishimi, Y., Komamura-Kohno, Y., You, Z., Omori, A., and Kitagawa, M. (2000) J. Biol. Chem. 275, 16235–16241
28. Ishimi, Y., and Komamura-Kohno, Y. (2001) J. Biol. Chem. 276, 34428–34433
29. Yao, W., and Garcia-Blanco, M. A. (1998) J. Biol. Chem. 273, 20629–20635
30. Hingorani, M. M., Washington, M. T., Moore, K. C., and Patel, S. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5012–5017
31. Poplawski, A., Grabowski, B., Long, S. E., and Kelman, Z. (2001) J. Biol. Chem. 276, 4931–4937
32. Loebri, G., Stenger, J. E., Ray, S., Parsons, R. E., Anderson, M. E., and Tegtmeier, P. (1991) J. Virol. 65, 3167–3174
33. Rose, P. E., and Schaffhauser, B. S. (1995) J. Virol. 69, 2842–2849
34. Dean, F. B., Borowiec, J. A., Ekl, T., and Hurwitz, J. T. (1992) J. Biol. Chem. 267, 14129–14137
35. Yan, H., Sato, T., and Tye, B. K. (1993) Genes Dev. 8, 944–957
36. Bird, L. E., Subramanya, H. S., and Wigley, D. B. (1998) Curr. Opin. Struct. Biol. 8, 14–18
37. Bujalowski, W., Klimowska, M. M., and Jezeewska, M. J. (1994) J. Biol. Chem. 269, 31350–31358
38. Sato, M., Gotow, T., You, Z., Komamura-Kohno, Y., Uchiyama, Y., Yabuta, N., Nojima, H., and Ishimi, Y. (2000) J. Mol. Biol. 300, 421–431

Models of the DNA Helicase Actions of the Mcm4/6/7 Complex—Two models, termed “inchworm” and “active rolling,” have been proposed for the actions of DNA helicases (2, 40). Both a Walker A mutation described previously (Table I) (9) and a zinc finger mutation described in this study resulted in reduced DNA binding activity, but showed differential effects on DNA helicase activity. This indicates that these segments are involved in interaction with DNA in distinct manners. The zinc finger mutation may alter the overall structure of the protein complex. The DNA binding activities (39). It should be noted that the N-terminal region of Mcm4 contains multiple serine/threonine residues, some of which may be phosphorylation sites of cyclin-dependent kinase and other kinases. It is an intriguing possibility that the interaction of Mcm with DNA and its helicase activity are regulated by phosphorylation of the N-terminal region of the Mcm4 protein. However, at the moment, we cannot rule out the possibility that the truncations caused alterations of the overall structure of the protein complex.

Both a Walker A mutation described previously (Table I) (9) and a zinc finger mutation described in this study resulted in reduced DNA binding activity, but showed differential effects on DNA helicase activity. This indicates that these segments are involved in interaction with DNA in distinct manners. The zinc finger mutation may alter the overall structure of the complex, affecting subunit assembly as well as DNA binding activity, which turned out to be stimulatory for DNA helicase. On the other hand, the Walker A mutant may be deficient in coupling of ATP hydrolysis to DNA unwinding, as shown in T7gp4 helicase (19).
Roles of Mcm7 and Mcm4 Subunits in the DNA Helicase Activity of the Mouse Mcm4/6/7 Complex
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