Interactions between the archaeal Cdc6 and MCM proteins modulate their biochemical properties

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ABSTRACT

The origin recognition complex, Cdc6 and the minichromosome maintenance (MCM) complex play essential roles in the initiation of eukaryotic DNA replication. Homologs of these proteins may play similar roles in archaeal replication initiation. While the interactions among the eukaryotic initiation proteins are well documented, the protein–protein interactions between the archaeal proteins have not yet been determined. Here, an extensive structural and functional analysis of the interactions between the *Methanothermobacter thermautotrophicus* MCM and the two Cdc6 proteins (Cdc6-1 and -2) identified in the organism is described. The main contact between Cdc6 and MCM occurs via the N-terminal portion of the MCM protein. It was found that Cdc6–MCM interaction, but not Cdc6–DNA binding, plays the predominant role in regulating MCM helicase activity. In addition, the data showed that the interactions with MCM modulate the autophosphorylation of Cdc6-1 and -2. The results also suggest that MCM and DNA may compete for Cdc6-1 protein binding. The implications of these observations for the initiation of archaeal DNA replication are discussed.

INTRODUCTION

Initiation of DNA replication requires the assembly of multi-protein complexes at the origin. In *Escherichia coli*, DnaA protein binds to *oriC* where, aided by additional proteins, it locally unwinds the origin [reviewed in (1)]. Then, ATP-bound DnaC associates with DnaB, the replicative helicase, and recruits it to the origin-DnaA complex to form a prepriming complex. Upon binding to the origin DNA, ATP bound to DnaC is hydrolyzed, releasing DnaC from the complex and activating the helicase (2). *In vitro*, interactions between DnaA and DnaB, and DnaB and DnaC, have been reported but no direct interactions between DnaA and DnaC were observed (3).

In eukarya, initiation starts with the assembly of a six-subunit origin recognition complex (ORC) at the origin, with ORC serving as a platform on which the pre-replication complex is assembled. In addition to ORC, the pre-replication complex includes the minichromosome maintenance (MCM) helicase, Cdc6, Cdt1 and several additional proteins. The release of the helicase and the initiation of DNA synthesis depend on the activity of several proteins, including Mcm10, geminin, Cdc45 and cell-cycle-dependent kinases [reviewed in (4)]. Using two-hybrid analysis, pull-down experiments and immunoprecipitation, interactions between many of these proteins were reported, including interactions between Cdc6, MCM and ORC [for example see (5)].

The initiation process in archaea is currently unknown. *In silico* analysis suggested that archaeal DNA replication proteins are more similar to those in eukarya than to those found in bacteria. However, the archaeal replication complexes contain fewer subunits than the eukaryotic homologs [reviewed in (6,7)]. Based on primary amino acid sequence analysis it was shown that most archaea contain a single MCM homolog and one or two Cdc6/ORC homologs (6,7). Some exceptions do exist and up to four MCM and nine Cdc6/ORC homologs have been identified in different archaeons. The eukaryotic Cdc6 protein shows amino acid sequence similarity to subunits of ORC (Orc1, 4 and 5), and it has not yet been determined whether the archaeal Cdc6/ORC homolog functions as ORC, Cdc6 or both. Hereafter, the archaeal Cdc6/ORC proteins will be referred to as Cdc6.

Biochemical studies with the MCM proteins from *Methanothermobacter thermautotrophicus* (8–15), *Sulfolobus solfataricus* (16–20), *Archaeoglobus fulgidus* and *Aeropyrum pernix* (21) revealed that the enzymes possess $3' \rightarrow 5'$ helicase activity, single-stranded (ss) and double-stranded (ds) DNA-binding activity, ssDNA and dsDNA translocation and a DNA-dependent ATPase activity.

The structure of the archaeal MCM complex is unclear. The MCM homologs of *S.solfataricus* (16,20), *A.fulgidus*

and *A.pernix* (21) form hexamers in solution. The *M.thermautotrophicus* enzyme appears to form dodecamers in solution (8–10) and a dodecamer was also suggested by the crystal structure (15) and biochemical studies (14) of the N-terminal portion of the protein. However, electron microscope reconstructions of the full-length *M.thermautotrophicus* MCM complex revealed hexameric (22), heptameric (23) and filamentous structures (24).

The archaeal MCM proteins consist of two main portions. The N-terminal region participates in protein multimerization and ssDNA binding (11,14,15,20) while the C-terminal part contains the helicase catalytic domain(s) (9,10,16).

A high-resolution 3D structure of the N-terminal portion of the *M.thermautotrophicus* MCM protein revealed a dumbbell-shaped double-hexamer (15). Each monomer folds into three distinct domains. Domain A, at the N-terminus, is mostly α-helical. Domain B has three β-strands and contains a zinc-finger motif. This motif was shown to participate in ssDNA binding (11,14). Domain C, positioned between domains A and B, contains five β-strands that form an oligonucleotide/oligosaccharide binding (OB) fold and connects the N-terminal portion of the enzyme to the catalytic region. The domain contains a β-finger shown to be involved in ssDNA and dsDNA binding (15,25). Domain C was also shown to be necessary and sufficient for MCM multimerization (14).

To date, only limited studies have been reported on the biochemical properties of the archaeal Cdc6 proteins. Studies on the enzymes from *M.thermautotrophicus* (12,26, 27), *S.solfataricus* (16–19,28), *A.fulgidus* (21) and *A.pernix* (29) show that the archaeal Cdc6 proteins can bind ssDNA and dsDNA. It was also found that inverted repeats located at the origins of replication (30) are better substrates for Cdc6 binding in comparison with random DNA sequences (27,28,31), and preferential binding to forked or bubble structures in comparison with ssDNA or dsDNA was also reported (18,21). In addition, the Cdc6 proteins were shown to inhibit MCM helicase activity when bound to ATP (12,17,19). ATP hydrolysis was not required for the inhibition (12). The proteins from different archaeons were shown to undergo autophosphorylation utilizing the γ-phosphate of ATP or dATP (12,17,19,26). The autophosphorylation is inhibited in the presence of ssDNA or dsDNA (26). However, the site of phosphorylation is currently unknown.

The 3D structure of the Cdc6 homologs from the archaeons *Pyrobaculum aerophilum* (32) and *A.pernix* (29) revealed the expected domains found in other members of the AAA+ superfamily of ATPases (33,34). In addition to the ATPase domains (domains I and II), the proteins contain a C-terminal winged-helix (WH) domain (domain III), which is present in Cdc6 proteins from all organisms. Amino acids substitutions and deletions within the WH domain demonstrated that the domain plays an important role in DNA binding (19,26,27,29).

The interactions among the archaeal initiation proteins are currently unknown. Therefore, a study was initiated to determine the interactions between the *M.thermautotrophicus* MCM and the two Cdc6 homologs identified in this organism (Cdc6-1 and -2). The data presented here demonstrate that both *M.thermautotrophicus* Cdc6-1 and -2 proteins interact with the N-terminal portion of MCM. These interactions are required for the regulation of MCM helicase activity by Cdc6. The interactions also modulate the autophosphorylation

of Cdc6-1 and -2. The data also suggest that MCM and DNA may compete for Cdc6-1 binding. This competition may play an important role in regulating the process of initiation of DNA replication.

MATERIALS AND METHODS

Materials

ATP and [γ-^{32}P]ATP were obtained from GE Bioscience, and oligonucleotides were synthesized by the Center for Advanced Research in Biotechnology DNA synthesis facility. The construction and purification of the *M.thermautotrophicus* MCM, Cdc6 and proliferating cell nuclear antigen (PCNA) proteins used in this study were described previously (12,24,35).

Methods

Generation of mutant proteins. Cdc6 mutants were generated using a PCR-based approach as previously described for the construction of MCM mutants (14) using plasmid containing the gene encoding the wild-type *M.thermautotrophicus* Cdc6-1 and -2 proteins (26). The 3D structures of the *P.aerophilum* and *A.pernix* Cdc6 proteins (29,32) served as the guide for the construction of the mutant proteins. The oligonucleotides used to generate the Cdc6 and MCM mutants are shown in Supplementary Table 1.

For the two-hybrid analysis, the various mutants were cloned into the pDBLeu and pPC86 vectors (Invitrogen) between the SalI and AatII sites, yielding fusion proteins to the GAL4 DNA binding (DB) or activation (AD) domains, respectively.

For protein expression in *E.coli*, the mutant proteins, containing six His residues upstream of the stop codon of MCM and at the N-terminus of Cdc6, were cloned into the pET-21a vector (Novagen) between the NdeI and XhoI sites. The Cdc6-1 and -2 proteins with maltose-binding protein (MBP) tags were generated by cloning the genes into an *E.coli* expression vector containing His$_6$-MBP recognition site at the N-terminus (36). Following expression in *E.coli*, the proteins were purified on a Ni-column.

The various MCM mutants containing a cAMP-dependent protein kinase recognition motif were generated by PCR using the mutant genes as template as described previously (37).

Two-hybrid analysis. For the two-hybrid analysis, pDBLeu and pPC86 vectors containing the various combinations of MCM and Cdc6 mutant genes were generated (see above). Plasmids encoding the AD and DB fusion proteins were co-transformed into yeast MaV203 cells (Invitrogen) according to the manufacturer's protocol. Cells were plated on complete supplement mixture (CSM) plates without Leu and Trp and grown for 3 days at 30°C. Colonies were streaked on CSM plates without Leu, Trp, and His and containing 10 mM 3-amino-1,2,4-triazole to suppress glycerol phosphate dehydratase, an enzyme involved in histidine biosynthesis. Plates were incubated at 30°C and replica cleaned after 24 h. Following further incubation at 30°C, the growth of yeast cells on these plates were monitored and scored every 24 h for 4 days. Growth indicates that the proteins fused to the AD and DB vectors interact. Interactions were also analyzed using

CSM plates without Leu, Trp and Ura as well as with a β-galactosidase assay for LacZ expression.

Far western dot-blot assay. Protein labeling for Far western analysis was performed as described previously (37) using 1.8 nmol of protein (as monomers) in a 100 μl reaction containing 20 mM Tris–HCl, pH 7.5, 15 mM magnesium acetate, 2 mM DTT, 100 mM NaCl, 15 μl (50 pmol) $[\gamma\text{-}^{32}P]ATP$ (3000 Ci/mmol, GE Bioscience) and 5 U of protein kinase A (Sigma) at 37°C for 60 min. The labeled proteins were purified from the unincorporated nucleotides using a sephadex G-50 gel filtration column equilibrated with reaction buffer.

Far western dot-blot assays were carried out using Minifold I (Schleiher and Schuell) apparatus by blotting 0.05, 0.15, 0.25, 0.5, 1.5 and 2.5 nmol or 0.05, 0.15, 0.5 and 1.5 nmol of protein (as monomers) onto nitrocellulose membrane (Schleicher and Schuell) pre-washed with 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate). Following blotting, the wells were washed three times with 0.5× SSC. The nitrocellulose membrane was then blocked by incubating in 1× TBST buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 3% Tween-20) containing 4% (w/v) non-fat dry milk for 18 h at 4°C. The blocked nitrocellulose membrane was washed three times (20 min each) with 50 ml of HYB buffer (20 mM HEPES-NaOH, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl and 10% glycerol) at 22°C. Hybridization was carried out with 300 pmol of ^{32}P-labeled proteins in 20 ml of HYB buffer for 3 h at 22°C. The membrane was washed three times (20 min each) with 50 ml HYB buffer at 22°C, air-dried and analyzed by phosphorimaging (Molecular Dynamics). The membrane was then cut and the radioactivity adsorbed by each dot in the membrane was measured by liquid scintillation counter. The specific activities of the labeled proteins used were: full-length MCM, 4.5 c.p.m./fmol; ΔA MCM, 3.5 c.p.m./fmol; ΔB MCM, 1.3 c.p.m./fmol; ΔC MCM, 1.8 c.p.m./fmol; N-ter, 30.6 c.p.m./fmol; PCNA, 80 c.p.m./fmol. All experiments were repeated three times and the average amount of ^{32}P-labeled proteins bound to each dot as well as representative blots are shown in Figure 2.

Protein pull-down assay. The pull-down assays were carried out by binding 2 μg of MBP-tagged Cdc6-1 or -2 proteins to 30 μl amylose resin (New England Biolabs) washed and equilibrated with 100 μl binding buffer containing 20 mM Tris–HCl, pH 7.5, at 22°C for 10 min. Following the binding of Cdc6 to the resin, 6 μg of MCM or PCNA proteins were added and the reaction was incubated for an additional 10 min at 22°C. The beads were then washed two times with 500 μl wash buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl and centrifuged at 4500 r.p.m. for 30 s. Proteins bound to the beads were eluted with 40 μl elution buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl and 50 mM maltose. The samples were then analyzed after adding 10 μl of 5× SDS loading buffer (250 mM Tris–HCl, pH 6.8, 500 mM DTT, 10% SDS, 0.5% Bromophenol blue and 50% glycerol), boiled and separated on 10% SDS–PAGE and visualized by Coomassie blue staining. A representative gel is shown in Figure 3.

DNA helicase assay. The substrate for helicase assays was made as described previously (13) by annealing a 25mer oligonucleotide 5′-CCGACGTGCCAGGCCGACGCGTCCC-3′ which was pre-labeled with $[\gamma\text{-}^{32}P]ATP$ and T4 polynucleotide kinase, to a 50mer oligonucleotide 5′-GGGACGCGTCG-GCCTGGCACGTCGGCCGCTGCGGCCAGGCACCCGA-TGGC-3′.

The substrate for duplex DNA translocation assays was made as previously described (13) by annealing a 61mer oligonucleotide 5′-(TTTG)$_9$CCGACGTGCCAGGCCGACGC-GTCCC-3′ which was pre-labeled with $[\gamma\text{-}^{32}P]ATP$, to two other oligonucleotides: a 25mer 5′-CCGACGTGCCAGGCC-GACGCGTCCC-3′ and a 50mer 5′-GGGACGCGTCGGCCT-GGCACGTCGGCCGCTGCGGCCAGGCACCCGATGGC-3′.

DNA helicase activity was measured in reaction mixtures (15 μl) containing 20 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA, 5 mM ATP, 10 fmol of ^{32}P-labeled DNA substrate (3000 c.p.m./fmol), and 0.3 pmol of MCM protein (as monomer). To determine the effect of the various Cdc6-1 and -2 mutant proteins on the MCM helicase activity, 0.3, 1.2 and 4.8 pmol of these proteins were added to the helicase assay. After incubation at 60°C for 1 h, reactions were stopped by adding 5 μl of 5× loading buffer (100 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% Bromophenol blue and 50% glycerol), and aliquots were loaded onto an 8% native polyacrylamide gel in 0.5× TBE (90 mM Tris, 90 mM boric acid and 1 mM EDTA) and electrophoresed for 1.5 h at 200 V. The helicase activity was visualized and quantitated by phosphorimaging. All helicase experiments were repeated three times and their averages with standard deviations are shown in Figure 4 together with representative gels.

Filter binding assay. Filter binding assays were performed either with 45mer ssDNA (MR163; 5′-TACATATGTAC-ATGGGTACATATGTACATGGGTACATATGTACAT-3′) or with dsDNA generated by annealing the MR163 oligonucleotide to its complementary sequence MR164 (5′-ATGT-ACATATGTACCCATGTACATATGTACCCATGTACAT-ATGTA-3′). DNA labeling and purification were performed as described previously (13).

Nitrocellulose filter binding assays were carried out by incubating 0.1, 0.3, 0.9 and 2.7 pmol of protein (as monomers) at 60°C for 10 min in a 20 μl reaction containing 20 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA, 1 mM ATP and 50 fmol of 5′ ^{32}P-labeled oligonucleotides. After incubation, the mixture was filtered through an alkaline-washed nitrocellulose filter (Millipore, HA 0.45 mm) (38), which was then washed with 20 mM HEPES-NaOH, pH 7.5. The radioactivity adsorbed to the filter was measured by liquid scintillation counting. Each experiment was repeated three times and the averages with standard deviations are shown in Figure 5.

Protein autophosphorylation. Protein autophosphorylation assays were performed as described previously (26) with 10 pmol of Cdc6 proteins in a reaction mixture (15 μl) containing 3.3 pmol of $[\gamma\text{-}^{32}P]ATP$, 25 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$ and 1 mM DTT in the presence or in the absence of 20 pmol of MCM ($K_{325}\rightarrow A$) protein. Following incubation for 20 min at 65°C, the reaction was stopped by adding 5 μl of 5× SDS loading buffer (250 mM Tris–HCl, pH 6.8, 500 mM DTT, 10% SDS, 0.5% Bromophenol blue and 50% glycerol), boiled, and the proteins were separated on 10% SDS–PAGE followed by Coomassie blue staining and autoradiography. The experiment was repeated four times. A representative gel and autoradiograph is shown in Figure 6.

RESULTS

MCM interacts with Cdc6-1 and -2 proteins

In vitro studies demonstrated that the *M.thermautotrophicus* Cdc6-1 and -2 proteins inhibit MCM helicase activity and it was, therefore, suggested that the proteins interact (12). Hence, interactions between the MCM and Cdc6-1 and -2 proteins were studied using two-hybrid analysis. The 3D structure of the N-terminal portion of *M.thermautotrophicus* MCM (15) and the structures of *P.aerophilum* and *A.pernix* Cdc6 proteins (29,32) were used as a guide for constructing mutant and truncated proteins.

Genes encoding various MCM- and Cdc6-derived proteins were generated using a PCR-based approach (see Materials and Methods) and cloned into the pDBLeu and pPC86 vectors (Invitrogen), resulting in fusion proteins containing the GAL4 DNA binding (DB) and activation (AD) domains, respectively. Different combinations of Cdc6 and MCM derivatives were analyzed for their ability to interact. The mutant proteins and the terminology used throughout the text are shown in Supplementary Table 2. Using this assay, Cdc6-1 showed clear and strong interactions with MCM (Figure 1A). However, no interaction could be detected between Cdc6-2 and MCM or Cdc6-1 and Cdc6-2 in the two-hybrid assay.

As shown in Figure 1A, the C-terminal catalytic domain of MCM is not required for the interaction with Cdc6-1 because the N-terminal part of the MCM molecule is sufficient for efficient binding. In addition, a protein with a mutation in the Walker-A motif ($K_{325} \rightarrow A$) of MCM, which is devoid of helicase activity (9,10) also strongly interacts with Cdc6. The data also suggest that domain C of the N-terminal region of MCM is required for Cdc6-1 binding, as proteins lacking either domain A (ΔA), domain B (ΔB) or both domains (ΔAB) are capable of binding to Cdc6-1. When domain C was removed from MCM (ΔC) no interaction with Cdc6 could be detected.

The data presented in Figure 1A suggest that the WH domain of Cdc6-1 is the main contact region to MCM. A truncated protein containing only the AAA$^+$ catalytic domains (domains I and II) did not interact with any MCM derivative (data not shown). In addition, proteins containing mutations in the Walker-A and -B motifs of Cdc6 retain their ability to interact with MCM, illustrating that an active ATPase domain is not required for MCM binding. It was also found that the WH domain alone is capable of interaction with the N-terminal portion of MCM. Interestingly, the WH domain did not interact with the full-length MCM protein. The presence of the catalytic domains of MCM may prevent access of the Cdc6-1 WH domain to the binding site on MCM when the AAA$^+$ domains of Cdc6 are missing. However, in the full-length Cdc6-1 protein, the WH domain is far from the AD domain of the fused protein and thus may have a better access to the binding site on domain C of MCM.

The two-hybrid analysis also suggests that DNA binding by MCM and/or Cdc6-1 is not required for interaction between the proteins. The β-finger and zinc-finger mutants of MCM bind Cdc6-1 as well as the intact protein (Figure 1A). The β-finger mutant cannot bind dsDNA (15) or ssDNA [(25); R. Kasiviswanathan and Z. Kelman, unpublished data], while the zinc-finger mutant is impaired in ssDNA (11) and dsDNA (R. Kasiviswanathan and Z. Kelman, unpublished
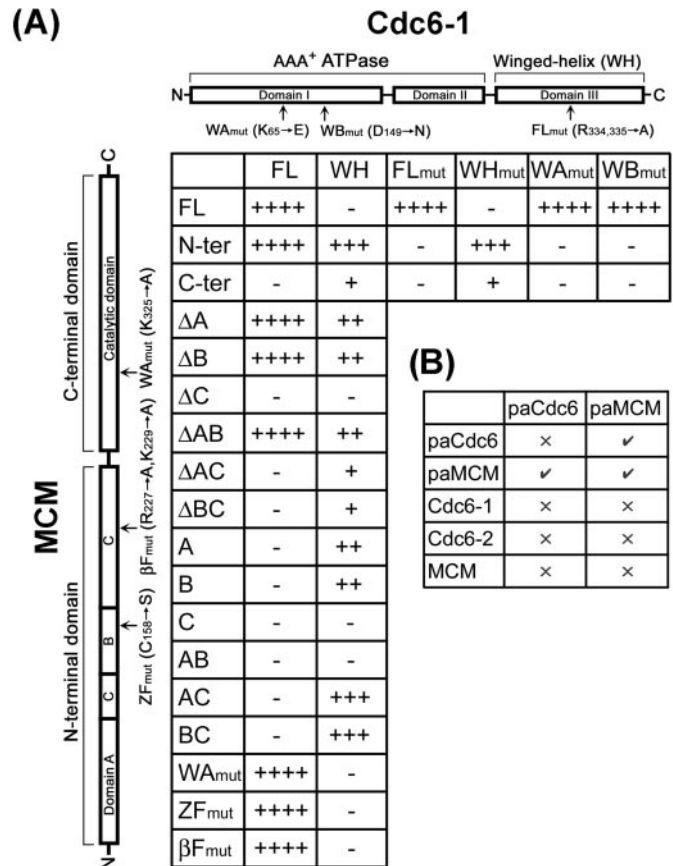

Figure 1. Cdc6-1 protein interacts with MCM protein in a two-hybrid analysis. A summary of the two-hybrid analysis of the interactions between the various *M.thermautotrophicus* (**A**) and *P.aerophilum* (**B**) MCM- and Cdc6-derived proteins, performed as described in Materials and Methods. In (A), cell growth observed after 24 h, ++++; 48 h, +++; 72 h, ++; 96 h, + and no growth, - are shown. In (B), 'tick mark' indicates cell growth after 2 days and 'cross' indicates that no growth was observed.

data) binding. A Cdc6-1 protein with mutations in the WH domain, which was shown to be devoid of DNA binding (27), retained MCM binding (Figure 1A).

To demonstrate that the interactions between MCM and Cdc6 are specific for the *M.thermautotrophicus* proteins, the single Cdc6 and MCM homologs from *P.aerophilum* were used as controls for the two-hybrid analysis. The genes encoding the *P.aerophilum* proteins were cloned into the pDBLeu and pPC86 vector and analyzed for their ability to interact with each other and with the *M.thermautotrophicus* enzymes. As shown in Figure 1B, although the *P.aerophilum* Cdc6 and MCM proteins interact with each other they failed to bind the *M.thermautotrophicus* enzymes. These observations strongly suggest species-specific interactions among the archaeal initiation proteins. Species specificity was previously shown by the ability of the *M.thermautotrophicus* Cdc6 to inhibit the *M.thermautotrophicus* MCM helicase activity, while the *P.aerophilum* Cdc6 could not (12).

To confirm the observation made with the two-hybrid analysis, and to determine whether Cdc6-2 is capable of binding MCM, several of the MCM and Cdc6-1 and -2 derived proteins were expressed and purified from *E.coli* (see Materials and Methods), and their ability to interact was determined using a Far western experiment (schematically described in
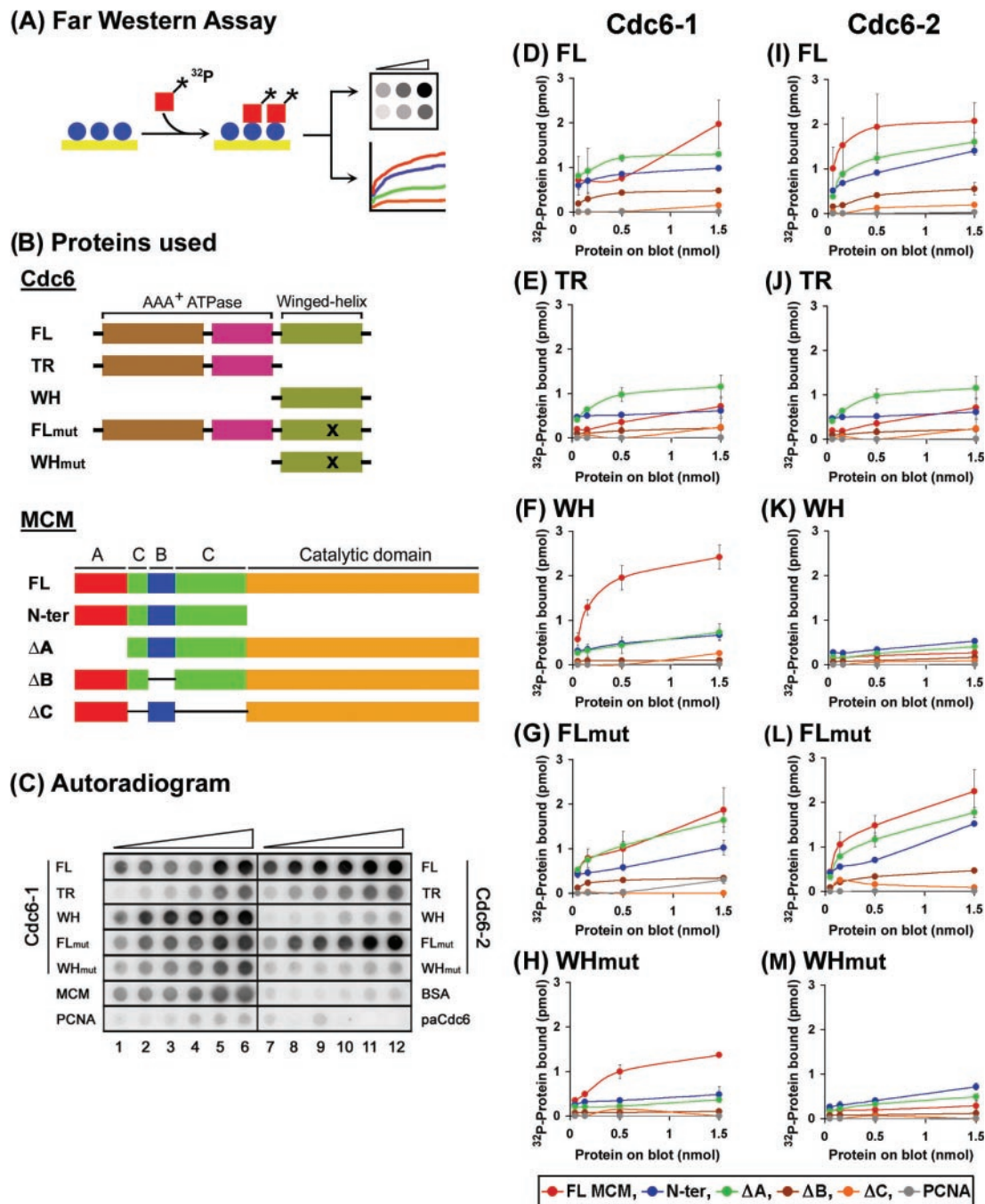
Figure 2. Cdc6 proteins interact with MCM protein in a Far western analysis. A Far western assay was performed as described in Materials and Methods with various concentrations of Cdc6-1 and -2 derived proteins and *M.thermautotrophicus* ^{32}P-labeled proteins as probes. (**A**) A schematic representation of the Far western dot blot assay. (**B**) The Cdc6 and MCM proteins used in the study. 'X', in the FLmut and WHmut of Cdc6 in (B), indicates the position of the WH mutations (Cdc6-1, $R_{334,335} \rightarrow A$ and Cdc6-2, $R_{337} \rightarrow A$). (**C**) A representative blot obtained using FL MCM as a probe. The amount of proteins used in the blot is lanes 1 and 7, 0.05 nmol; lanes 2 and 8, 0.15 nmol; lanes 3 and 9, 0.25 nmol; lanes 4 and 10, 0.5 nmol; lanes 5 and 11, 1.5 nmol; lanes 6 and 12, 2.5 nmol. (**D–M**) The averages of three independent experiments (with error bars) for the amounts of the various probes used bound to the Cdc6-1 and -2 derived proteins. The colors used are red, FL MCM protein; blue, N-ter MCM; green, ΔA MCM; brown, ΔB MCM; orange, ΔC MCM; gray, PCNA. The colors used are also shown at the bottom of the figure.

Figure 2A). The various amounts of the Cdc6 proteins (0.05–2.5 nmol) were absorbed onto a nitrocellulose filter and probed with ^{32}P-labeled full-length MCM or its derivatives at a concentration of 15 nM (300 pmol of proteins in 20 ml reaction volume) (see Materials and Methods). An example of the results obtained with the full-length MCM is shown in Figure 2C. The graphs in Figure 2D–M summarize the results

of three independent experiments performed with the different probes. The level of interaction detected is very low as only a few pmols of labeled proteins are interacting with nmol amounts of proteins on the filter. This may be explained by the low concentrations of MCM proteins used in the experiment and/or the low affinity of Cdc6 and MCM. This is supported by the inability of our group, and others, to detect

direct interactions between the proteins in a sizing column or glycerol gradient.

Confirming the observation made with the two-hybrid analysis (Figure 1A), the full-length MCM protein interacts efficiently with the full-length Cdc6-1 protein (Figure 2C and D) and its WH domain (Figure 2F) in the Far western experiment. In fact, the interaction between MCM and the WH domain of Cdc6-1 appears stronger than the interaction with the full-length Cdc6-1 enzyme. It is possible that the region of interaction on the WH domain is exposed when the catalytic domain is removed from the Cdc6-1 molecule, resulting in better binding.

In the Far western assay, the full-length MCM also interacts with Cdc6-2 (Figure 2I). The region(s) participating in MCM binding, however, are somewhat different than those of Cdc6-1. While the Cdc6-1 WH domain binds to MCM, only weak binding could be detected between MCM and the WH domain of Cdc6-2 (compare Figure 2F and K). In fact, appreciable binding could be detected only between MCM and either the full-length Cdc6-2 (Figure 2I) or its mutant form (Figure 2L, FLmut).

Next, several truncated MCM proteins containing the cAMP-dependent protein kinase recognition motif were purified, labeled and analyzed for their ability to interact with the various Cdc6 proteins, as described above for the full-length MCM enzyme. As shown in Figure 2D–M, proteins containing only the N-terminal part of MCM, or proteins missing domain A (ΔA) or domain B (ΔB), all interact with the full-length Cdc6-1 and -2 proteins and their mutant forms (FLmut) (Figure 2D, G, I and L). The proteins also interact with the truncated and WH domains of Cdc6-1 and -2, but to a lesser extent (Figure 2E, F, J and K). Protein lacking domain C (ΔC), however, did not interact with any of the Cdc6-derived enzymes (Figure 2D–M). Similar to the two-hybrid analysis, these data show that the N-terminal portion of MCM plays a major role in Cdc6 interaction and that domain C may be required for binding. However, protein containing only the N-terminal domain shows weaker binding to Cdc6 than the full-length enzyme, suggesting a minor role for the catalytic domain of MCM in Cdc6 binding.

As described above, the MCM protein lacking domain A (ΔA) binds to Cdc6-1 and -2 as well as the N-terminal portion of the molecule (Figure 2D and I). The protein lacking domain B (ΔB), however, binds more weakly to both Cdc6-1 and -2 than either the N-terminal part or the ΔA protein (Figure 2D and I). These results may suggest that the Cdc6 binding site is located in the region connecting domains B and C. This possibility would also explain the observation that protein lacking domain C cannot bind Cdc6.

Several controls demonstrate that the interactions observed in the Far western assays are specific for Cdc6 and MCM. BSA did not interact with the full-length MCM (Figure 2C) or its truncated forms (data not shown). In addition, a Cdc6 homolog from *P.aerophilum* also failed to interact with the *M.thermautotrophicus* MCM (Figure 2C). These observations are consistent with the two-hybrid analysis that also failed to detect interactions between the *M.thermautotrophicus* and *P.aerophilum* proteins (Figure 1B). These results demonstrated that although all archaeal Cdc6 proteins are similar in structure and primary amino acid sequences (39), their interactions with MCM are species-specific.

As an additional control, a blot similar to that used in Figure 2C was probed with ^{32}P-labeled *M.thermautotrophicus* PCNA protein (Figure 2D–M) (35). PCNA is a good negative control for MCM-interacting proteins. Both proteins are ring-shaped homomultimers that encircle DNA and both have a similar charge distribution, with positive charged residues in the central cavity and negative charged residues on the outer surface (15,40). As shown in Figure 2D–M, PCNA did not bind to Cdc6-1 and -2, further demonstrating that the interactions between Cdc6 and MCM are specific. The PCNA protein also did not interact with either MCM or BSA, which were also used as controls (data not shown).

The experiments described in Figures 1 and 2 were performed with either immobilized proteins or in a yeast heterologous system. Therefore, in order to demonstrate that purified *M.thermautotrophicus* MCM and Cdc6 interact in solution, pull-down experiments were performed. Untagged MCM proteins were incubated with MBP-tagged Cdc6-1 or -2 proteins bound to amylose resin (Figure 3). As shown in Figure 3, MCM can be pulled down by its association with either Cdc6-1 (Figure 3, lane 7) or -2 (Figure 3, lane 9). In the absence of Cdc6 protein no MCM was observed in the pull-down fraction (Figure 3, lane 3), demonstrating that the results obtained are due to MCM interaction with Cdc6 and not from non-specific interactions with the amylose resin. As an additional control, the *M.thermautotrophicus* PCNA protein was used in a similar experiment. PCNA could not be pulled down by either Cdc6-1 or -2 (Figure 3, lanes 11 and 13).

Cdc6–MCM interactions are required for efficient regulation of MCM helicase activity by Cdc6 protein

After establishing that Cdc6 and MCM interact, the effect of the interactions on MCM helicase activity was determined. It was previously shown that Cdc6-1 and -2 inhibit MCM
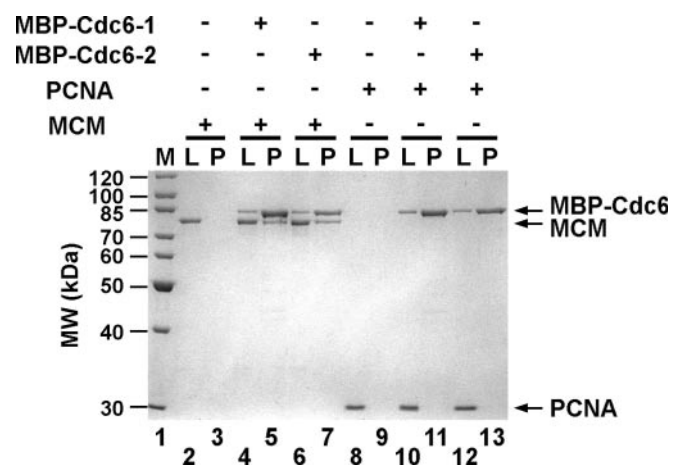

Figure 3. Cdc6-1 and -2 proteins interact with MCM in solution. Protein pull-down assays were performed as described in Materials and Methods by binding 2 μg of MBP-tagged Cdc6-1 or -2 proteins to amylose resin in the presence of 6 μg of untagged MCM or PCNA proteins. Lane 1, molecular weight marker; lanes 2 and 3, MCM alone; lanes 4 and 5, MCM and MBP-Cdc6-1; lanes 6 and 7, MCM and MBP-Cdc6-2; lanes 8 and 9, PCNA alone; lanes 10 and 11, PCNA and MBP-Cdc6-1; lanes 12 and 13, PCNA and MBP-Cdc6-2. Lanes 2, 4, 6, 8 and 10 contain 10% of the reaction mixture and are marked by 'L'. Lanes 3, 5, 7, 9 and 11 contain the proteins eluted from the amylose resin and are marked by 'P'.
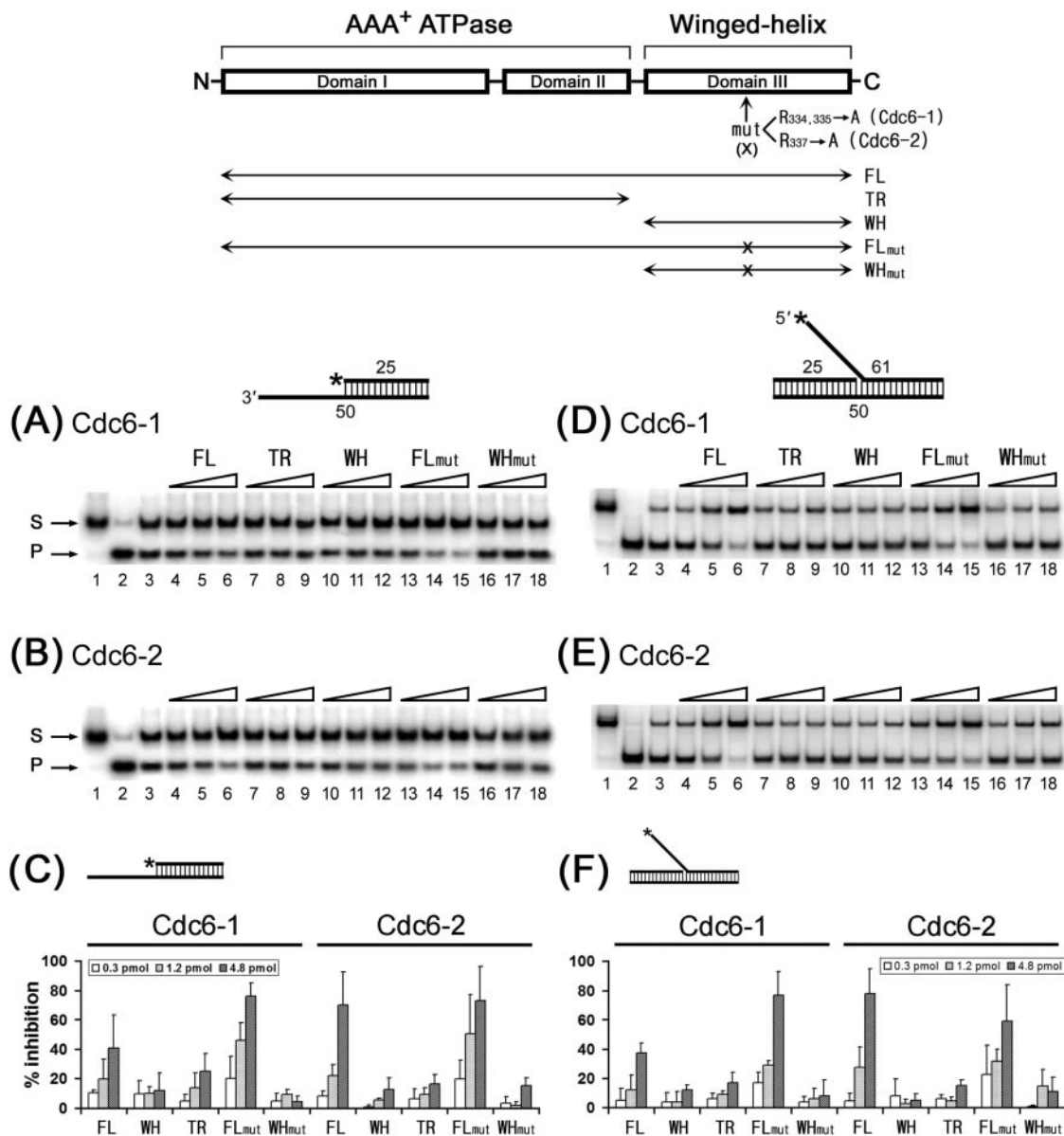
Figure 4. Cdc6–MCM interaction is required for the inhibition of MCM translocation along DNA. MCM helicase translocation along ssDNA (**A–C**) and dsDNA (**D–F**) was assayed as described in Materials and Methods in the presence of 0.3 pmol MCM (as monomer) and increasing amounts of Cdc6-1 and -2 proteins and their derivatives. (A, B, D and F) show representative gels. Lane 1, substrate only; lane 2, boiled substrate; lane 3, no Cdc6; lanes 4, 7, 10, 13 and 16, 0.3 pmol of Cdc6 protein as monomer; lanes 5, 8, 11, 14 and 17, 1.2 pmol of Cdc6 protein as monomer; lanes 6, 9, 12, 15 and 18, 4.8 pmol of Cdc6 protein as monomer. ^{32}P-labeled strands are marked by an asterisk. (C and F) summarize the percent inhibition of MCM translocation (C) and duplex translocation (F) observed in the presence of the various Cdc6 proteins. Error bars represent the standard deviation calculated from 3 experiments. Top: Schematic representation of the Cdc6-1 and -2 proteins used in the study.

helicase activity (12). However, it is not yet clear whether Cdc6–MCM or Cdc6–DNA interactions play the major role in this inhibition. Therefore, the various Cdc6 mutant proteins were studied for their ability to inhibit MCM helicase activity. As shown in Figure 4, both full-length Cdc6-1 and -2 protein inhibit helicase activity (Figure 4A and B, compare lanes 4–6 with lane 3; see also Figure 4C). As shown previously (12), Cdc6-2 inhibits MCM helicase activity better than Cdc6-1 (compare lanes 4–6 in Figure 4A and B; see also Figure 4C).

Is DNA binding by Cdc6 required for MCM inhibition? It was shown that substitution of two Arg residues (Arg$_{334}$ and Arg$_{335}$) by Ala in the recognition helix of the WH domain of

Cdc6-1 completely abolished dsDNA binding (27). In Cdc6-2 there is only a single Arg residue (Arg$_{337}$) in a similar location and, therefore, this residue was also replaced by Ala. These mutations were generated in the full length and in the WH domain constructs of Cdc6-1 and -2 (see diagram on top of Figure 4). The genes encoding these mutant proteins were cloned into pET-21a (Novagen) for protein expression in *E.coli*.

As shown in Figure 5, while Cdc6-2 binds efficiently to both ssDNA and dsDNA (Figure 5C and D), Cdc6-1 binds weakly to dsDNA and not at all to ssDNA (Figure 5A and B). Although the WH domain was reported as the main interaction
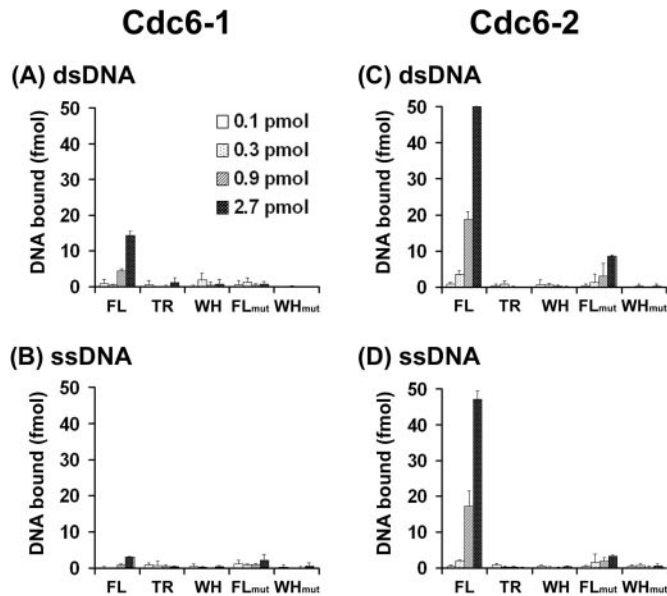
Cdc6-1

Cdc6-2

Figure 5. An intact WH domain of Cdc6 is needed for DNA binding. Filter binding assays were performed as described in Materials and Methods using ^{32}P-labeled single-stranded or dsDNA oligonucleotides in the presence of 0.1, 0.3, 0.9 and 2.7 pmol of proteins (as monomer). The averages with standard deviations of three experiments are shown. (**A**) dsDNA binding of Cdc6-1 proteins; (**B**) ssDNA binding of Cdc6-1 proteins; (**C**) dsDNA binding of Cdc6-2 proteins; (**D**) ssDNA binding of Cdc6-2 proteins.

region between the archaeal Cdc6 proteins and dsDNA (26,27), the intact WH domain fails to interact with DNA (Figure 5A and C). This may be because the domain is misfolded or that the AAA^+ domains are also required for dsDNA binding. The AAA^+ domains are required for Cdc6-2 binding to ssDNA (26).

As previously reported (27), Cdc6-1 protein with a mutation in the WH domain failed to interact with dsDNA (Figure 5A). Although a protein with a similar mutation in Cdc6-2 retained some dsDNA binding ability, this was substantially reduced in comparison with the wild-type enzyme (Figure 5C).

To determine whether DNA binding by Cdc6 is required for the inhibition of MCM helicase activity, the WH mutant proteins were studied for their effect on MCM helicase activity. As shown in Figure 4, both Cdc6-1 and -2 full-length enzymes containing mutations in the WH motif (FLmut) are capable of inhibiting the helicase (Figure 4A and B, lanes 13–15; see also Figure 4C), illustrating that DNA binding is not essential for inhibition. Interestingly, the mutant Cdc6-1 protein appears to be a better inhibitor than the wild-type enzyme (Figure 4A, compare lanes 13–15 with lanes 4–6; see also Figure 4C). The WH domains of the Cdc6 proteins were shown to be required for interaction with MCM (Figures 1 and 2) and for DNA binding (26,27). These interactions may compete and thus when Cdc6-1 interaction with DNA is abolished, tighter interaction with MCM can occur, resulting in better inhibition. Such competition may play a role during the initiation process at the origin DNA (see Discussion). Furthermore, though the WH protein of Cdc6-1 interacted with the MCM, no efficient helicase inhibition could be observed. This could be because interaction between the full-length Cdc6-1 protein and MCM may dissociate the MCM complex [as was previously suggested (8)] while the WH domain of Cdc6-1 protein may not.

It was shown that the *M.thermautotrophicus* MCM as well as the eukaryotic helicase can translocate along duplex DNA (13,41). It was, therefore, suggested that dsDNA translocation by the replicative helicase may play a role during the initiation and/or elongation phases of DNA replication (42). As Cdc6 proteins play an essential role in the initiation process in eukarya, and probably in archaea, the effects of the interactions between Cdc6 and MCM on duplex translocation by MCM were studied.

As shown in Figure 4D–F, and similar to the results with helicase translocation along ssDNA (Figure 4A–C), both full-length Cdc6 proteins inhibit duplex translocation by the helicase. Similar to inhibition of ssDNA translocation, Cdc6-2 is a better inhibitor in comparison with Cdc6-1 (Figure 4, compare lanes 4–6 in D and E; see also Figure 4F). Cdc6-1 mutant protein, devoid of DNA binding, is a much better inhibitor of duplex translocation by the helicase than the full-length protein (Figure 4D, compare lanes 13–15 with lanes 4–6; see also Figure 4F).

Cdc6–MCM interaction modulates Cdc6 autophosphorylation

After demonstrating that interaction between Cdc6 and MCM is involved in the regulation of helicase activity, the effect of the interaction on Cdc6 autophosphorylation was determined. To date, the only biochemical activity besides DNA binding shown for the archaeal Cdc6 proteins is the ability to undergo autophosphorylation in the presence of γ-ATP (12,17,19,26). Although the level of phosphorylation *in vitro* is low (26) it has been reported for all archaeal proteins studied. Therefore, it was proposed that autophosphorylation might play a regulatory role during initiation (6,7,26). It was also hypothesized that Cdc6–MCM interactions may regulate the phosphorylation activity during assembly of the helicase around DNA at the origin (6,26). The regulation of autophosphorylation by DNA binding was previously demonstrated (26).

Thus, the effect of the interactions between Cdc6 and MCM on Cdc6 autophosphorylation was analyzed (Figure 6). A mutant form of MCM in which Ala replaced Lys325 was used in order to prevent ATP hydrolysis by MCM that would limit the available ATP for the Cdc6 autophosphorylation reaction. As shown in Figure 6, the presence of MCM modulates the autophosphorylation of both Cdc6 full-length proteins. However, the effect of the interaction with MCM has the opposite effect on Cdc6-1 and -2. While binding of MCM stimulates the phosphorylation of Cdc6-1 (Figure 6A and B, compare lane 4 with lane 3), the interaction with Cdc6-2 inhibits autophosphorylation (Figure 6A and B, compare lane 10 with lane 9). It is possible that the WH domain of Cdc6-1 contains the phosphorylation site, and in solution it is packed against the rest of the molecule and prevents it from being efficiently phosphorylated. MCM binding to the WH domain of Cdc6-1 (Figures 1 and 2) may expose the residue, resulting in better phosphorylation. In support of this idea, a truncated form of Cdc6-1, missing the WH domain, cannot be phosphorylated (Figure 6A and B, lanes 5 and 6). This is in contrast to Cdc6-2 in which a truncated protein retains the ability to autophosphorylate (Figure 6A and B, lanes 11 and 12) (26). Also, mutant forms of both full-length proteins phosphorylate to a lesser extent than the unmutated enzymes. It is possible that
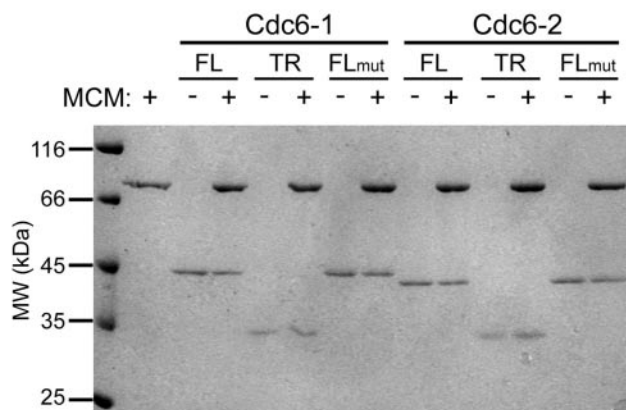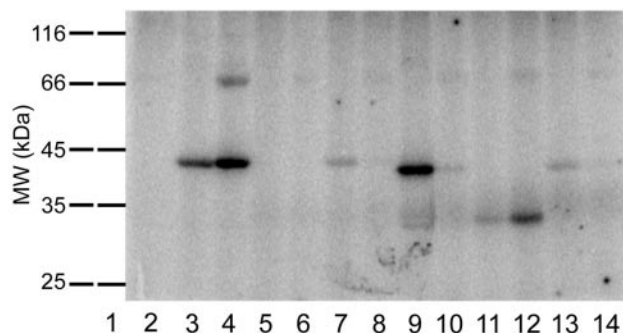
(A) Coomassie

(B) Autoradiogram

Figure 6. Cdc6 autophosphorylation is regulated by MCM binding. Cdc6 autophosphorylation reactions were performed as described in Materials and Methods in a reaction mixture (15 μl) containing 10 pmol of Cdc6 protein and 3.3 pmol of [γ-^{32}P]ATP in the absence (lanes 3, 5, 7, 9, 11 and 13) or in the presence (lanes 4, 6, 8, 10, 12 and 14) of 20 pmol MCM. The autophosphorylation reactions were carried out for 20 min at 65°C. Following incubation, the proteins were separated by 10% SDS–PAGE and visualized by Coomassie blue staining (**A**) and autoradiography (**B**). Lane 1, molecular mass (kDa); lane 2, MCM alone; lanes 3 and 4, Cdc6-1 full-length protein; lanes 5 and 6, Cdc6-1 truncated proteins; lane 7 and 8, Cdc6-1 full-length protein with mutant WH domain; lanes 9 and 10, Cdc6-2 full-length; lanes 11–12, Cdc6-2 truncated proteins; lane 7 and 8, Cdc6-2 full-length protein with a mutated WH domain. A representative gel is shown.

the mutation affects the WH structure in such a way that the phosphorylation site is not accessible.

The reason for the opposite effect of MCM on Cdc6-1 and -2 phosphorylation is unknown. However, in light of the prevailing hypothesis that one Cdc6 homolog is needed for origin recognition while the other acts in MCM loading (6), one would expect to see differences in the interactions between the two proteins and MCM and this may result in different effects on phosphorylation. When a helicase loading assay is developed, this hypothesis can be tested.

DISCUSSION

In both eukarya and archaea, the mechanism by which the MCM helicase is assembled around the DNA at the origin is not yet understood. However, in both systems, the prevailing notion is that the Cdc6 protein, in conjunction with ORC (or its

functional homolog in archaea), plays an essential role in helicase loading. In addition, it is believed that the mechanism will be similar to the assembly of the *E.coli* DnaB helicase at *oriC*.

As a first step toward the elucidation of helicase assembly at the archaeal origin, the interactions between the Cdc6 and MCM proteins from the archaeon *M.thermautotrophicus* were studied and demonstrated that the proteins interact and that the association between the two proteins regulates their respective enzymatic properties.

What is needed for Cdc6–MCM interaction?

Two-hybrid and Far western analysis demonstrated an interaction between MCM and the two Cdc6 homologs identified in the *M.thermautotrophicus* genome. The study also identified the domains needed for the interactions. Although both Cdc6 homologs are similar in primary amino acid sequence (39) and may have similar structure and domain organization (29,32), they appear to utilize different regions for MCM binding. While Cdc6-1 binds MCM predominantly via the WH domain, this domain of Cdc6-2 does not interact with the helicase. Only the full-length Cdc6-2 protein showed appreciable MCM binding. This is similar to the observations made with a Cdc6 homolog from the archaeon *S.solfataricus*, in which an indirect assay suggested that the WH domain of one of the three Cdc6 homologs found in the organism is not required for MCM binding (18). In addition, it was shown that although the three Cdc6 homologs of *S.solfataricus* are similar in primary amino acid sequence, the proteins have different functions (28).

The interaction between Cdc6-2 and MCM may also be similar to that of DnaA and DnaB in *E.coli*. It was shown that, while DnaA binds to the DnaB helicase via a region located at the N-terminal part of the AAA$^+$ catalytic domains (43), the origin recognition domain is at the C-terminal region of the molecule (44). Similarly, it was demonstrated that the eukaryotic Cdc6 protein also interacts with MCM via the AAA$^+$ catalytic domains, which are separated from the WH domain (45).

The archaeal MCM proteins can be divided into two main portions, the N-terminal region, needed for protein multimerization and DNA binding, and the C-terminal AAA$^+$ catalytic domain, responsible for catalytic activity (9). The data presented here suggest that the major contact between MCM and Cdc6 is via domain C of the N-terminal portion of MCM. Neither domain A, which has been suggested to play a regulatory role, nor domain B, needed for ssDNA binding, is essential for interaction with Cdc6. In addition, neither ssDNA nor dsDNA binding by Cdc6 or MCM is needed for the interactions, as mutant proteins devoid of DNA binding retain the ability to interact with MCM, and DNA was not present in the Far western analysis.

Do the *M.thermautotrophicus* Cdc6-1 and -2 proteins play different roles during the initiation process?

The *M.thermautotrophicus* Cdc6-1 and -2 proteins are proposed to have different functions during initiation. It has been suggested that one protein is the origin binding protein, and thus is the functional homolog of the eukaryotic ORC and bacterial DnaA, while the other is the functional homolog

of the eukaryotic Cdc6 and bacterial DnaC, and participates in helicase loading (6).

In silico analysis of different archaeal Cdc6 proteins suggests that they belong to two distinct subgroups, referred to as groups I and II (29,39). However, it is not yet clear whether these two groups have different functions. It is also not yet clear whether they have different structures, as the two structures solved to date are of proteins belonging to subgroup II (29,32). As part of the difference between the two subgroups lies in the WH domains, it was suggested that proteins of the two subgroups might bind DNA differently. While the *M.thermautotrophicus* Cdc6-2 [subgroup II (29)] can bind both ssDNA and dsDNA (Figure 5), Cdc6-1 [subgroup I (29)] can bind only dsDNA (27) (Figure 5). The observation that the two *M.thermautotrophicus* Cdc6 proteins interact differently with DNA substrates and utilize different regions for MCM binding supports the hypothesis of different roles for these proteins during the initiation process.

It was proposed that the autophosphorylation of Cdc6 proteins may regulate helicase loading and/or be regulated by the initiation process (26). The observation that Cdc6-1 and -2 autophosphorylation is regulated by MCM supports this hypothesis. Furthermore, the observation that autophosphorylation of one protein is stimulated by MCM binding while that of the other is strongly inhibited may suggest different roles for Cdc6-1 and -2 during the initiation process. Whether this is the case remains to be seen.

Does a switch mechanism between Cdc6, MCM and DNA regulate the initiation of DNA replication?

Studies have demonstrated that the archaeal Cdc6 proteins can regulate MCM helicase activity (12,17,19). There are several possible mechanisms for this inhibition. Direct binding of Cdc6 to MCM may prevent helicase movement along the DNA. The interaction might also destabilize the MCM complex or destabilize MCM interactions with DNA. Alternatively, binding of Cdc6 to the ssDNA and/or the duplex part of the DNA substrate may prevent helicase translocation along the DNA. Previous studies using full-length Cdc6 proteins from different archaeons (12) suggested that direct interactions between Cdc6 and MCM are required for the inhibition. These studies illustrated that when the Cdc6 and MCM are from the same organism, efficient inhibition could be observed. Only limited inhibition was detected when Cdc6 and MCM from different species were tested. As all Cdc6 proteins studied retained their DNA-binding activity, these observations suggested that protein–protein interactions are needed for helicase inhibition.

The use of the large number of mutant proteins described in this study takes this analysis further. The data show that Cdc6-1 and -2 have different inhibitory effects on MCM translocation. While Cdc6-2 is an efficient inhibitor of both ssDNA and dsDNA translocation by the helicase, only ∼50% of the inhibition observed with Cdc6-2 could be detected with Cdc6-1. The study also clearly illustrates that DNA binding by Cdc6 is not the predominant factor for the inhibition of MCM activity. Cdc6–DNA interaction may play some role, however. Furthermore, mutant forms of Cdc6-1 and -2, impaired in DNA binding, inhibit MCM helicase activity as well or better than the wild-type enzyme. This is an interesting and potentially

important observation. It suggests that MCM and DNA may compete for Cdc6 binding. When Cdc6 cannot bind DNA, it binds MCM more tightly and thus efficiently inhibits the helicase activity. It may suggest the possibility of a switch mechanism mediated by Cdc6 during the initiation process in which Cdc6 binds to MCM, brings it to the DNA at the origin and then switches to DNA binding, releasing the helicase, which can then associate with the DNA. It is also possible that only one of the Cdc6 proteins (the helicase loader) is involved in the switch, while the other forms the DNA structure (replication bubble) on which the helicase will be loaded.

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