Regulation of Minichromosome Maintenance Helicase Activity by Cdc6*

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Genetic studies, together with amino acid and structural similarities to the clamp loaders of DNA polymerase sliding clamps, have suggested that the Cdc6 protein may function as a loader for the eukaryotic replicative helicase, the minichromosome maintenance (MCM) complex. Thus, Cdc6 may act as the functional homologue of the bacterial DnaC that utilizes ATP hydrolysis to assemble the DnaB helicase at the origin. This report shows that the helicase activity of an MCM homologue from the archaeon Methanothermobacter thermautotrophicus is inhibited in the presence of the Cdc6 homologues. This inhibitory activity is dependent, as for DnaC, on ATP binding to Cdc6. Moreover, an intact Cdc6 winged helix domain is required for efficient inhibition. Two-hybrid analyses indicated that MCM and Cdc6 interact and that the interaction is mediated by the winged helix domain. Analysis of Cdc6 and MCM homologues from several archaea exhibited differences in the inhibitory activity suggesting divergence in function in Cdc6 and MCM homologues among the archaea.

In all organisms, the initiation of DNA replication requires the assembly of multiprotein complexes at the origin. In Escherichia coli, the origin recognition factor, DnaA, binds to oriC where, aided by additional proteins, it unwinds the origin. The DnaB helicase complexed to ATP-bound DnaC is then recruited to the origin to form a prepriming complex. On binding to the origin DNA, the DnaC ATP is hydrolyzed, releasing DnaC and activating the DnaB helicase (1, 2).

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 (pre-RC) is assembled. The pre-RC includes, in addition to ORC, 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 several cell cycle-dependent kinases (3, 4).

The eukaryotic MCM is a family of six essential proteins (MCM2–7) with highly conserved amino acid sequences. In vivo and in vitro studies have revealed that in addition to the heterohexameric form, the proteins can form several additional complexes with different combinations of the MCM proteins (5). Biochemical studies with the various complexes have shown that a dimeric complex of the MCM4,6,7 heterotrimer contained 3′–5′-DNA helicase activity, ssDNA binding, and DNA-dependent ATPase activities, whereas its interactions with either MCM2 or MCM3,5 inhibited the helicase activity (5–7). All six proteins, however, were shown to be essential for replication fork progression (8), and mutational analyses have shown that physical interactions between specific members of the two subgroups (MCM4,6,7 and MCM2,3,5) are required for efficient ATPase activity (9).

The eukaryotic Cdc6 has been shown to be an essential component of the pre-RC, required for the recruitment of the MCM helicase to the origin (10). It has also been shown to modulate ORC structure and its DNA binding properties (11). Following MCM loading and the initiation of S phase, Cdc6 is phosphorylated by CDKs, transported to the cytoplasm, and eventually degraded in a ubiquitin-dependent manner (12). Cdc6 protein shows significant similarity to the clamp loaders of DNA polymerase processivity factors (13), which assemble a ring-shaped sliding clamp that associates with the replicative polymerase on primed DNA and serves as its processivity factor (14). The similarities between Cdc6 and the clamp loaders of DNA polymerase, together with the genetic and in vivo observations, suggest that Cdc6 may act as a loading factor for MCM (10, 13).

In the 3rd domain of life, the archaea, DNA replication proteins resemble those of eukarya but assume somewhat simpler forms. Most archaeal species that have been sequenced contain at least one Cdc6 homologue and one MCM homologue (15, 16). In the archaea Methanothermobacter thermautotrophicus (mt) one MCM and two Cdc6 homologues (mtCdc6-1 and mtCdc6-2) have been identified. Biochemical studies with the mtMCM protein demonstrated that the enzyme possesses properties similar to those of the eukaryotic MCM4,6,7 complex, including ssDNA binding, ATPase activity stimulated by DNA, and 3′–5′-helicase activity that is dependent on ATP or dATP hydrolysis (17–20). The protein was shown to form double hexamers in solution (17, 18) an observation supported by the three-dimensional structure of the N-terminal half of the protein (21).

The three-dimensional structure of the Cdc6 homologue from the archaean Pyrobaculum aerophilum (pa) revealed the ex-
pected domains found in other members of the AAA\(^+\) superfamily of ATPases (22). The protein also contains a C-terminal winged helix (WH) domain (domain III), which has been identified in Cdc6 proteins from all organisms. Studies with the archaeal homologues of Cdc6 revealed the need for an intact WH domain for dsDNA interactions (16, 23).

To date, only one biochemical study has been reported on mtCdc6-1 and -2 proteins (23). It was shown that the proteins interact with both ssDNA and dsDNA and that dsDNA binding is mediated by the WH motif at the C terminus. It was also demonstrated that the proteins undergo autophosphorylation on Ser residues utilizing the γ-phosphate of ATP or dATP. This autophosphorylation is inhibited in the presence of ssDNA and dsDNA.

Although helicase loading and the initiation process are not yet well understood in eukaryota, the well-characterized bacterial system may provide a model for the loading mechanism. In eukaryota, the Cdc6 protein is presumed to be the helicase loader functioning in a fashion similar to the bacterial DnaC (24), which assembles the DnaB helicase around the origin DNA in an ATP-dependent manner. When complexed with DnaB, DnaC inhibits the ATPase and helicase activities of DnaB, but on ATP hydrolysis by DnaC and assembly of the helicase around origin DNA, DnaC is released from both DnaB and the origin (25, 26). ATP binding to DnaC is not necessary for its interaction with DnaB but is needed for the inhibition of DnaB activity (27). This inhibition is thought to prevent DnaB from functioning in an origin-independent manner. Thus, if the Cdc6 protein is the helicase loader, it may function in a manner similar to DnaC, and its interaction with MCM should also inhibit the helicase.

In this study, the effects of mtCdc6-1 and -2 on mtMCM helicase activity were examined. The results show that the helicase activity of mtMCM is inhibited in the presence of either mtCdc6-1, mtCdc6-2, or both simultaneously. The data demonstrate that the inhibition is dependent on ATP binding by mtCdc6. Moreover, the mtCdc6 WH motif is required for the inhibitory activity. It also appears that the inhibition is different for the different C-terminal domain of Cdc6 and MCM proteins, suggesting functional divergence in the archaeal Cdc6 and MCM homologues.

EXPERIMENTAL PROCEDURES

Materials

Labeled and unlabeled ATP and dATP were obtained from Amer sham Biosciences. ssM13mp19 was from New England Biolabs, and oligonucleotides were synthesized by Integrated DNA Technologies. mtMCM and paCdc6 were purified as described previously (23), and the purification of Sulfobolus sulfataricus (ss) MCM will be described elsewhere.

Methods

Constructing the mtCdc6-1 and -2 Mutants—The mtCdc6-1 and -2 single amino acid mutants were generated with the QuickChange Site-directed Mutagenesis kit (Stratagene) using a pET-16b plasmid (Nova gen) containing the wild-type gene as a template. Following mutagen esis, the genes were sequenced to ensure that no additional mutations were created. The truncated forms of mtCdc6-1 and -2 were generated using PCR in which the 3′ primers contained a stop codon after residue 305 (mtCdc6-1) or 291 (mtCdc6-2), as suggested by Liu et al. (26).

Expression and Purification of mtCdc6 Recombinant Proteins—All proteins were overexpressed and purified to near-homogeneity from E. coli (DE3) pLysS cells (Novagen) using a modification of a protocol provided by Dr. James Berger. Cells harboring the different plasmids were grown at 37 °C in Luria-Bertani (LB) medium in the presence of appropriate antibiotics. When the culture reached an A\(_{600}\) of 0.5, protein expression was induced by incubation in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h, after which time the cells were harvested. The cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, and 20% glycerol. After sonication, the cell debris was removed by centrifugation at 4 °C, and the lysate was bound to the appropriate amount of Ni\(^2+\)-beads with gentle shaking for 1 h at 4 °C. Following binding, the mixture was poured into a column and washed with lysis buffer followed by a wash with lysis buffer containing 10 mM imidazole at 4 °C. The column was moved to 22 °C, and all subsequent elution steps were carried out at that temperature. The column was washed with 10 column volumes of 100 mM and 2 column volumes of 150 mM imidazole in elution buffer containing 40 mM Tris-HCl (pH 8.0), 0.4 M potassium acetate, and 20% glycerol. The proteins were then eluted in 0.5–1-mL fractions with increasing concentrations of imidazole (200, 250, 300, and 350 mM) in the same elution buffer. Each protein eluted in a different imidazole concentration in this range. The protein in the eluted fractions was visualized with SDS-PAGE, and protein concentrations were measured by Bradford (Bio-Rad) with bovine serum albumin (BSA) as the standard. Protein samples were flash-frozen in liquid nitrogen and stored at −70 °C.

Preparation of Substrates for Helicase Assays—Three oligonucleotides were used for the preparation of the helicase substrates. These included a 100-mer, 5′-CTGCCCGCTTTTCCAGTTGGGAAACTGTCG- TGGCACCGTCG(TTGTT)_3′, corresponding to the longer strand of partial duplex substrates (100-mer); an 80-mer, 5′-GGTTTTGGCGACTGCGGC- ACGACAGTTCTTCCAGGGAAAGGGGCCGAC-3′ (80-mer); and a 5′- GCACCTGGCCAGCGACATTTCCCCGTTGCGAGAAAGGGGCCGAC-3′ (40-mer). Annealing of the 40-mer to the 100-mer or to ssM13mp19 results in substrates with a 3′ single-stranded overhang, referred to as a flat substrate. Annealing of the 80-mer to the 100-mer or to ssM13mp19 results in substrates with both 3′ and 5′ single-stranded overhangs and is referred to as a forked substrate.

To prepare the helicase substrate, the short oligonucleotides (either the 40- or the 80-mers) were labeled with [γ-\(^{32}\)P]ATP and T4 polynucleotide kinase and annealed either to the 100-mer oligonucleotide or to ssM13mp19. Following annealing, the M13 substrate was purified over a 1-mL Sephacryl HS-300-HR column, and the oligonucleotide substrate fragments were purified by electrophoresis on a polyacrylamide gel.

DNA Helicase Assay—DNA helicase activity was measured in a reaction mixture (15 μL) containing 20 mM Tris-HCl (pH 8.5), 10 mM MgCl\(_2\), 2 mM diithiothreitol, 100 μg/mL BSA, 5 mM ATP or dATP, 10 fmol of \(^{32}\)P-labeled DNA substrate (3,000 cpm/fmol), and 25 ng of MCM (0.05 pmol as hexamer). After incubation at 60 °C for 1 h, the reaction was 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% polyacrylamide gel in 0.5× TBE (45 mM Tris, 4.5 mM borate acid, 0.5 mM EDTA) and electrophoresed for 30 min at 200 V and 4 °C.

The extent of helicase activity was quantitated by PhosphorImager (Amersham Biosciences) and presented as the percentage of product released from the substrate. The inhibitory effects of Cdc6 were determined by comparing the product released by the helicase in the presence and absence of the Cdc6 proteins as indicated in the figure legend.

Protein Phosphorylation—Protein phosphorylation assays were performed with 500 ng of protein in a reaction mixture containing 3.3 pmol of [γ-\(^{32}\)P]ATP, 25 mM Hepes-NaOH (pH 7.5), 5 mM MgCl\(_2\), 1 mM dithiothreitol, and 500 ng of BSA. The samples were incubated for 20 min at 65 °C in the presence or absence of 0.33 mM ATP or dATP. Following incubation, the proteins were separated on SDS-PAGE followed by Coomassie Blue staining and autoradiography.

Two-hybrid Analyses—For the two-hybrid analyses, the genes encoding both AD and DB fusion proteins were cloned into the pDBLeu vector (Invitrogen). The GAL4 DNA binding domain (DB) (pDBLeu-mtCdc6, pDBLeu-mtCdc6d, pDBLeu-mtCdc6T, pDBLeu-paCdc6, and pDBLeu-MCM, respectively). The gene encoding mtMCM was also cloned into the pPC86 (Invitrogen) resulting in a fusion protein with the GAL4 DNA binding domain (DB) (pDBLeu-mtCdc6, pDBLeu-mtCdc6d, pDBLeu-paCdc6, and pDBLeu-MCM, respectively). Plasmids encoding for 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 2–3 days at 30 °C. Colonies were streaked on CSM plates minus Leu, Trp, and His and containing 10 μM 3-amino-1,2,4-triazole to suppress yeast growth. Phosphate dehydratase, an enzyme involved in histidine biosynthesis. Colonies were also streaked on CSM plates minus Leu, Trp, and Ura. Plates were incubated at 30 °C for 2–3 days. Growth on these plates indicates that the proteins fused to the AD and DB interact.
RESULTS

mtCdc6 Proteins Inhibit the Helicase Activity of mtMCM—As a first step in determining whether Cdc6 is the helicase loader, the ability of the archaean Cdc6 to inhibit MCM helicase activity was determined. As shown in Fig. 1A, both mtCdc6-1 and -2, individually (lanes 4–6 and 8–10) and in combination (lanes 12–14), inhibited MCM helicase activity in a concentration-dependent manner (compare lanes 4–6, 8–10, and 12–14 to lanes 3, 7, and 11). The molar ratios of mtCdc6:mtMCM in the experiments shown in Fig. 1 ranged from 1.5 to 15. However, unlike DnaC, which was shown to stimulate the helicase activity of DnaB at a ratio of 1:1 and to inhibit it at higher molar ratios (26, 27), neither inhibition nor stimulation was seen when lower ratios (0.013–1.5) of mtCdc6:mtMCM were used (data not shown).

Although both proteins inhibited helicase activity, mtCdc6-2 was a consistently better inhibitor than mtCdc6-1 in all substrates used (Fig. 1B and data not shown). Notably, the combined inhibition of the two proteins was at the level of mtCdc6-1, which, although a less effective inhibitor of helicase activity, may be a better competitor for interaction with MCM. The two proteins may also act as heterodimers, with mtCdc6-1 suppressing the inhibitory activity of mtCdc6-2. Differential scanning calorimetry experiments indicated that mtCdc6-2 may dimerize in solution, and in vivo and in vitro studies of eukaryotic Cdc6 have also suggested that the Cdc6 proteins may form oligomers (12, 29). Although all experiments were performed with both proteins, only the results with mtCdc6-2 will be presented, unless otherwise specified.

ATP Binding by mtCdc6-2 Is Required for Inhibition of mtMCM Helicase Activity—ATP binding was found to be necessary for the inhibition of helicase activity by DnaC, whereas ATP hydrolysis was found to release that inhibition and activate DnaB (27). This could be demonstrated because DnaB and DnaC can utilize different nucleotides, i.e. DnaB can function with CTP but DnaC does not (27). In the presence of CTP, DnaB did not inhibit DnaB helicase activity, even at high DnaC concentrations, and DnaC-ATP–γS inhibited DnaB helicase activity better than DnaC-ATP. However, unlike DnaB and DnaC, mtMCM and mtCdc6 can only utilize ATP or dATP (17, 23). Thus, the requirement for ATP binding and/or hydrolysis for the inhibition of MCM helicase activity was determined using wild-type mtCdc6-2 protein as well as proteins with mutations at the Walker-A (Lys71 – Glu) and Walker-B (Asp146 → Asn) sites.

mtCdc6-1 and -2 exhibit extremely weak ATPase activity, and their ability to hydrolyze ATP has been demonstrated indirectly by their ability to autophosphorylate (23). On the basis of mutational analyses in other members of the AAA+ family of ATPases and the autophosphorylation assays of mtCdc6, it appears that the Walker-A mutant of mtCdc6-2 can neither bind nor hydrolyze ATP, whereas the Walker-B mutant can bind ATP but has reduced ATPase activity. As shown in Fig. 2A, the mtCdc6-2 Lys → Glu mutant is a much less efficient inhibitor of helicase activity than the wild-type protein or the Asp → Asn mutant (A, compare lanes 7–9 to lanes 4–6 and 10–12). This suggests that ATP binding but not hydrolysis is needed for the inhibitory activity of mtCdc6-2. In addition, just as DnaC-ATP–γS inhibited DnaB activity better than DnaC-ATP, mtCdc6 Asp → Asn inhibited mtMCM activity better than the wild-type protein (Fig. 2A, compare lanes 10–12 to lanes 4–6; see also C), suggesting that its ability to bind ATP but its lower level of ATP hydrolysis resulted in a more efficient inhibition of the helicase. These results are further supported by the use of dATP instead of ATP in the reaction.

As shown in Fig. 2, A and B, wild-type mtCdc6-2 inhibited mtMCM helicase activity to a similar extent with either ATP or dATP (lanes 4–6, see also C). As expected, the inhibition by the Lys → Glu mutant was the same whether ATP or dATP was used (A and B, lanes 7–9; see also C), as neither nucleotide can bind this mutant. However, the inhibitory behavior of the Asp → Asn mutant with ATP (A, lanes 10–12) differed from that with ATP. Although this mutant inhibited the helicase more efficiently when ATP was used (A, lanes 10–12, see also C), its inhibitory effect was diminished when dATP was used (B, lanes 10–12), with helicase inhibition intermediate between the wild-type and the Lys → Glu mutant (Fig. 2, A–C), suggesting a lower affinity for the deoxyribose.

To determine whether the reduced inhibitory effect is indeed due to the inability of the Asp → Asn mutant to interact efficiently with dATP, an autophosphorylation assay was performed in the presence or absence of unlabeled nucleotides (Fig. 2D). It was previously shown that the archaean Cdc6 proteins can undergo in vitro autophosphorylation on Ser residues using the γ-phosphate of ATP or dATP (16, 23). It was postulated that the enzyme has to bind the nucleotide and to sever the interactions between the β- and γ-phosphates prior to the transfer of the γ-phosphate to the Ser residue (23). Whereas an excess of either ATP or dATP inhibits the autophosphorylation of the wild-type protein (compare lane 2 to lanes 3 and 4), only ATP could effectively inhibit the Asp → Asn mutant (compare lane 5 to lanes 6 and 7). The Lys → Glu mutant does not autophosphorylate, as it does not bind any nucleotide (lanes 8–10). These results strongly suggest that the mtCdc6-2 Asp →

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Z. Kelman, unpublished observations.
Asn mutant has a reduced ability to bind dATP, possibly due to structural changes in the Walker-B site of the mutant protein. Both DnaC and human (h) Cdc6 show different affinities for ATP and dATP. DnaC was shown to have a 2–3 order of magnitude lower affinity for dATP compared with ATP (30). Similarly, hCdc6 showed little direct binding to dATP (29). A suggested explanation for the differences between the effect of ATP and dATP on the Asp → Asn mutant of mtCdc6-2 comes from an examination of the crystal structure of the paCdc6-2 (28). It was shown that the 2'-OH group of the nucleotide ribose participates in local interactions with an Arg residue located between the sensor I and sensor II regions of the protein (28). However, these interactions are disrupted with dATP. In addition, the crystal structure of paCdc6-2 shows that the acidic groups of the Walker-B motif (D/D/EXXX) stabilize nucleotide binding. Thus, although the wild-type protein with intact Walker-A and -B sites may compensate for the reduced affinity of the deoxyribose, the Asp → Asn mutant with a weakened Walker-B site may no longer efficiently bind dATP. Taken together, these experiments demonstrate that, similar to the observation in E. coli, efficient inhibition of MCM helicase activity requires ATP binding but not hydrolysis by the Cdc6 proteins.

The Winged Helix Domain of Cdc6 Is Needed for Efficient Inhibition of Helicase Activity—The three-dimensional structure of paCdc6-2 protein revealed the presence of a WH fold at the C terminus of the protein (28). Subsequent biochemical studies demonstrated that the WH domain of several archaeal Cdc6 proteins is involved in interactions with dsDNA (16, 23). The need for the WH domain in the inhibition of helicase activity was examined with truncated forms of mtCdc6-2 and -2, in which this domain was removed. As shown in Fig. 3, the inhibition of helicase activity by the truncated forms of mtCdc6-2 was lower than that of the full-length proteins (compare B to A, E to D; see also C and F). This was true for the truncated wild-type as well as the truncated Walker-A and -B mutants. Similar results were obtained with mtCdc6-1 (data not shown). These results suggest that dsDNA binding by the Cdc6 proteins may be required for efficient inhibition of helicase activity. It is also possible, however, that the WH domain participates in the interactions with MCM. Indeed, two-hybrid analyses showed that mtCdc6 and mtMCM interact (Fig. 4), as has also been found in budding yeast (32), and that the interaction is mediated by the WH domain (Fig. 4; compare sector 8 to sector 7). Although the inhibition by the truncated forms is at about the level of inhibition of the Lys → Glu mutant (Fig. 3, C and F), it is unlikely that the lower inhibitory activity of the truncated protein is due to the inability to bind ATP, as it has been shown to autophosphorylate better than the full-length protein (23).

Direct Interactions between mtMCM and mtCdc6—Two-hybrid analyses performed with mtCdc6-1 and mtMCM revealed an interaction between the two proteins (Fig. 4). As shown in Fig. 4 (sector 7) the mtCdc6 interacts with mtMCM. The specificity of the interaction was determined in sector 9. In contrast to mtCdc6, the Cdc6 homologue from P. aerophilum (paCdc6) did not bind to mtMCM. In addition, the possibility of an indirect interaction via a bridging protein in the two-hybrid screen is unlikely, given the differences between eukaryotic and archaeal proteins. Furthermore, an intact WH domain is needed for the interactions between the proteins, as the truncated form of mtCdc6-1 failed to interact with mtMCM (sector 8). These observations are consistent with the inability of the truncated form of Cdc6 to inhibit mtMCM helicase activity (Fig. 3). It was shown previously (16, 23) that this domain was important in interactions with dsDNA. Taken together with
the need for an intact WH domain for dsDNA binding (23), the data presented here suggest that the domain is important in both DNA binding and in interaction with the helicase.

Helicase Activity and Inhibition on Different Substrates—As shown in Fig. 5A, the activity of the mtMCM depends on the substrate used. Activity was higher with a linear substrate compared with ssM13, and with both linear and circular DNA, helicase activity was higher with a forked substrate. This is consistent with both bacterial and eukaryotic helicases. DnaB was shown to require a forked substrate for efficient unwinding (33, 34), and the processivity of the eukaryotic MCM4,6,7 was significantly enhanced by forked structures on which double hexamers were assembled (35). As mtMCM was shown to form double hexamers (17, 18, 21), it is possible that it has lower activity on a flat substrate because it may not be able to load efficiently as a double hexamer onto this substrate.

The inhibition of helicase activity by wild-type and mutant mtCdc6 was also tested on the different substrates (Fig. 3). Although the Lys → Glu mutant and truncated proteins were less efficient in inhibiting helicase activity overall, there were differences in the extent of inhibition with different substrates. When a flat substrate was used, the Lys → Glu mutant inhibited about 50% as well as the wild-type and Asp → Asn mutant proteins (Fig. 3A, compare lanes 7–9 to lanes 4–6 and 10–12, see also C). However, when a forked substrate was used, the inhibition by the Lys → Glu mutant was substantially reduced (~25% inhibition in comparison to that of the wild-type and Asp → Asn mutant) (D, compare lanes 7–9 to lanes 4–6 and 10–12, see also F).

Likewise, when the truncated proteins were used, substantially less inhibition was observed on a forked substrate in comparison to a flat substrate (compare C to F). Similar results were obtained with mtCdc6-1 (data not shown).

Phylogenetic Differences in Cdc6 Function—The archaeal domain consists of two main kingdoms, the euryarchaeota and the crenarchaeota, with several differences in their replication apparatus (15, 36). One of the differences is in the structure of MCM, at least in the two archaeal MCMs studied to date. Whereas the crenarchaeotal ssMCM is hexameric in solution (37), the euryarchaeotal mtMCM is double hexameric (17, 21). These structural differences suggest possible different interac-

3 A. T. McGeoch, Z. Kelman, and S. D. Bell, unpublished observation.
Regulation of MCM Helicase Activity by Cdc6

Mechanisms of Helicase Inhibition by Cdc6—Several (overlapping) mechanisms of inhibition are possible, including binding of Cdc6 to MCM and to DNA, thus preventing the helicase from translocating along the DNA, conformational change or paralysis in MCM, or destabilization of the MCM interaction with DNA. mtCdc6 may inhibit mtMCM helicase activity by direct interactions with ssDNA, thus preventing MCM binding. It appears that this is not the case, however, and that an interaction with MCM may also be needed. Cdc6 by itself interacts with DNA poorly, as filter binding assays and gel filtration failed to detect a strong interaction (data not shown). It is clear, however, that mtCdc6-1 and -2 interact with DNA in some fashion, as the autophosphorylation of these proteins is inhibited by ssDNA and dsDNA (23). mtCdc6 may exhibit cryptic DNA binding, as was demonstrated for DnaC, which did not bind DNA by itself, but only when complexed with DnaB (31). The requirement for an intact WH motif for efficient inhibition also supports the notion that the inhibition is not simply due to ssDNA binding. This motif was shown to be involved in dsDNA but not ssDNA binding (39). Also, when reactions were staged, i.e. substrate and MCM were incubated prior to the addition of Cdc6, or when substrate and Cdc6 were incubated prior to the addition of MCM, no significant differences in helicase inhibition could be observed (data not shown).

Direct interactions between mtCdc6 and mtMCM may be required for the inhibition of helicase activity (Fig. 7). Data presented here with a two-hybrid screen demonstrated an interaction between mtMCM and mtCdc6 (Fig. 4). Furthermore, a truncated protein that cannot bind MCM also cannot efficiently inhibit the helicase activity. Similar MCM-Cdc6 interactions have also been suggested for the eukaryotic proteins by genetic studies (10) and two-hybrid analysis (32). This observation will be similar to the inhibition observed in bacteria in which direct protein-protein interactions between DnaB and ORC are altered by mutants.
DnaC are needed for the inhibition of the helicase activity.

The possibility that helicase inhibition by Cdc6 requires both mtMCM and DNA binding is suggested by the observation that the WH domain, which has been shown to be involved in protein-protein interactions (Fig. 4) as well as dsDNA binding (39), is required for efficient inhibition. Cross-linking studies in bacteria showed that when DnaB is complexed with DnaC, only DnaC is cross-linked to DNA. Thus DnaC appears to inhibit DnaB helicase activity by suppressing or altering DNA binding by the helicase (31). Similarly, Cdc6 may bind to MCM and to DNA simultaneously, destabilizing MCM binding to DNA. Such a requirement for DNA binding prior to protein-protein interactions has been shown for other replication proteins (40).

mtCdc6 may also alter the conformation of mtMCM or inhibit the conformational changes necessary for mtMCM activity. DnaB hexamers can adopt two different architectures of $C_3$ and $C_6$ symmetry (41, 42), but binding of DnaC appears to freeze the helicase in the $C_3$ architecture (43). mtMCM has been shown to form double hexamers (17–19, 21), but electron microscopy images revealed heptameric structures, although some hexamers were also observed (44). It is possible that these two forms are in equilibrium and that binding to mtCdc6 may "freeze" mtMCM in the heptameric state. These heptamers may be unable to assemble around the DNA and/or to translocate along it, and only the removal of mtCdc6 will result in an active hexameric ring. A somewhat similar situation has been observed with the Thermus thermophilus RuvB protein, which forms a heptameric structure that converts to a hexamer upon DNA binding (45). As mtMCM can bind both ssDNA and dsDNA (21), it is also possible that the heptamers encircle dsDNA whereas the hexamers bind ssDNA.

Are mtCdc6-1 and -2 the Functional Homologues of ORC and Cdc6?—Cdc6 proteins share amino acid similarities with subunits of ORC. It is not yet clear whether the archaeal homologues are functional homologues of Cdc6, ORC, or both. In M. thermautotrophicus there are two homologues, and it was thought that one may be the functional homologue of the eu-

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**Fig. 5.** mtMCM helicase activity on various substrates. A, under "Experimental Procedures" with 25 ng of mtMCM (0.05 pmol as hexamer), 10 fmol of each substrate (as indicated). The amount of primer displacement is shown with the error bars representing the S.D. calculated from four independent experiments. B, models for helicase loading on flat and forked substrates. Only one hexamer can load onto the ss portion of the flat substrate in the proper 3'-5' polarity, whereas two hexamers can load with opposite polarity onto the ss portions of the forked structures.

**Fig. 6.** Kingdom differences in Cdc6 inhibition of MCM helicase activity. Helicase activity assays were performed as described under "Experimental Procedures" with 25 ng (0.05 pmol as hexamer) of mtMCM (lanes 3–9) or ssMCM (lanes 10–16), 10 fmol of flat substrate for 1 h at 60 °C in the presence of increasing amounts of mtCdc6-2 (lanes 4–6 and 11–13), or paCdc6 (lanes 7–9 and 14–16). Lane 1, substrate only; lane 2, boiled substrate; lane 3, no Cdc6; lanes 4, 7, 11, and 14, 25 ng (0.55 pmol as monomer); lane 5, 8, 12, and 15, 75 ng (1.66 pmol as monomer); lanes 6, 9, 13, and 16, 225 ng (5 pmol as monomer) of Cdc6 proteins. The percent inhibition in comparison to MCM activity in the absence of Cdc6 protein is indicated (%). S, substrate; P, product.
karyotic ORC and the other of Cdc6 (16, 17, 46). Although the two proteins share a high degree of amino acid sequence similarity and were suggested to have similar structure (28), the data presented here support the hypothesis that the two proteins have diverged in function, i.e. mtCdc6-2 inhibits MCM helicase activity more efficiently than mtCdc6-1. A phylogenetic analysis of origin-binding proteins in the three domains also showed that the two proteins have diverged (47). In addition, in M. thermautotrophicus, the origin is located upstream of the gene encoding the mtCdc6-1 protein (48). In bacteria it was shown that the origin-binding protein, DnaA, is often located in the vicinity of the origin (49), and a similar observation has also been made in archaea (50).

Alternatively, the two proteins may be redundant in function, or they may act cooperatively to prepare the origin for MCM binding, as was suggested for the eukaryotic ORC and Cdc6 proteins (38). In M. thermautotrophicus and other archaea with one or two Cdc6 homologues, Cdc6 may be combining the functions of both ORC and Cdc6, and the proteins may also act as dimers or higher oligomers. This was suggested by the study of the Pyrococcus abyssi cell cycle, which found that Cdc6 stayed bound at the origin, unlike eukaryotic Cdc6, but similarly to the eukaryotic ORC proteins (51).

**MCM Regulation by Cdc6 in Other Archaea**—There are large disparities in the numbers of Cdc6 genes in different archaeal species. Although most have one or two homologues, several organisms have none, whereas others contain up to nine (16, 52). The reasons for the large number of Cdc6 proteins in some organisms are not yet clear. It is possible that each protein has a unique function or that they are redundant and all have similar roles. It is also possible that organisms with multiple homologues more closely resemble the eukary, in which six different polypeptides form the active ORC. In light of the data presented here, it will be of interest to determine whether all, or only a subset, of archaeal Cdc6 proteins can interact with and inhibit MCM activity, and whether they function in origin recognition or in MCM loading or both.

To date most archaea have been found to contain a single MCM homologue (15, 16). Exceptions do exist, however. For example, the Methanocaldococcus jannaschii genome contains four MCM homologues, whereas Methanopyrus kandleri and Methanosarcina acetivorans have two. Intriguingly, the genomes of M. jannaschii and M. kandleri do not have a clear homologue of Cdc6. Thus the role of regulating helicase activity may reside in other proteins. However, as both organisms contain more than one MCM homologue, the additional MCM proteins may provide regulatory functions, similar to the eukarya, in which three of the six MCM proteins (MCM2,3,5) are thought to be regulatory (5). It is clear, however, that in eukarya more proteins are needed for the process in addition to ORC and Cdc6 (3, 4). Thus, in archaea, with fewer proteins required for the initiation process, the Cdc6 homologues may have a more extensive role in MCM regulation in comparison to the eukaryotic Cdc6. In addition, the eukaryotic Cdc6 proteins have a short half-life and may thus have a limited role in MCM loading, whereas in archaea where Cdc6 is a stable and abundant protein throughout the cell cycle (51), it may have additional regulatory functions.

Phylogenetic analyses show that the archaeal Cdc6 homologues and the eukaryotic ORC and Cdc6 proteins have diverged (47). Nevertheless, given their sequence similarity to the archaeal proteins, it is likely that the eukaryotic proteins retain some aspects of archaeal function, albeit supplemented by additional proteins and larger complexes. Future in vitro and in vivo studies should determine the true similarities and differences between the effects of the archaeal and eukaryal Cdc6 proteins on MCM activity.
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