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A CDC6-like Factor from the Archaea *Sulfolobus solfataricus* Promotes Binding of the Mini-chromosome Maintenance Complex to DNA*

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The archaeal replication apparatus appears to be a simplified version of the eukaryotic one with fewer polypeptides and simpler protein complexes. Herein, we report evidence that a Cdc6-like factor from the hyperthermophilic crenarchaea *Sulfolobus solfataricus* stimulates binding of the homohexameric MCM-like complex to bubble- and fork-containing DNA oligonucleotides that mimic early replication intermediates. This function does not require the Cdc6 ATP and DNA binding activities. These findings may provide important clues to understanding how the DNA replication initiation process has evolved in the more complex eukaryotic organisms.

Living organisms have evolved different strategies to recruit and load DNA helicases at the replication origins, and accessory factors, referred to as helicase loaders, are required to accomplish this task (1, 2). The helicase loading process was best characterized at the molecular level in two bacterial systems: *Escherichia coli* (3) and *Bacillus subtilis* (4). The *E. coli* replicative homohexameric DNA helicase DnaB associates with its loader, DnaC, forming a 6:6 complex that is recruited at the chromosomal replication origin (*oriC*) by the initiator protein DnaA (5). DnaB is then released in an active form, and this initiates formation of two replication forks that extend in opposite directions from *oriC* (6). Because *E. coli* DnaB forms stable hexamers in solution that are unable to efficiently self-load onto single-stranded DNA, it was proposed that its loading by DnaC is carried out by a ring-breaking mechanism (1). A similar ring-opening/closing mechanism was demonstrated to take place during loading of the *E. coli* phage T7 gp4 DNA helicase/primase, but in this case no accessory loading factor is required (7). In *B. subtilis*, the replicative DNA helicase DnaC forms homohexamers in solution in the presence of ATP, but without ATP the hexameric form readily dissociates into monomers. The preformed hexameric DnaC helicase is unable to unwind DNA and instead must be assembled from monomers around DNA by a loading system that consists of two proteins, DnaI and DnaB (4). A ring-forming mechanism was also proposed for the *E. coli* bacteriophage T4 helicase gp41 (8) and for the simian virus 40 large tumor antigen, but in this latter case a separate helicase loader is not required (1, 9).

It is now well established that DNA replication factors of Archaea are more similar in sequence to those found in Eukarya than to the analogous proteins of Bacteria (10). Analysis of the sequenced genomes revealed that the Archaea kingdom contains homologs of certain eukaryal initiation factors (such as Cdc6 and MCM), whereas evident homologs of some other initiation factors are not present at all (*e.g.* Cdt1, MCM10, and Cdc45). The hyperthermophilic crenarchaea *Sulfolobus solfataricus* (11) was found to possess a MCM-like complex (SsoMCM)[1] (12) and three Cdc6 homologs (SsoCdc6-1, -2, and -3) (13–15). The biological function of these proteins has not yet been clearly defined. Furthermore, it was found that the chromosome of *S. solfataricus* possesses three active DNA replication origins (15, 16). Two of them are located in front of the genes coding for SsoCdc6-1 (*oriC1*) and SsoCdc6-3 (*oriC2*). Whereas *oriC2* is bound by all three *S. solfataricus* Cdc6 factors, *oriC1* is bound only by SsoCdc6-1 and SsoCdc6-2. It was proposed that SsoCdc6-1 and SsoCdc6-3 promote initiation of DNA replication because they are present in G_1 and S phases, whereas SsoCdc6-2 may act as a negative regulator because it seems to be present only in G_2 phase *Sulfolobus* cells (15).

In this work we focused our attention on SsoCdc6-2 among the three *S. solfataricus* Cdc6 factors, because we have already investigated the biochemical properties of this protein (13). In fact, we previously reported that SsoCdc6-2 is a monomer in solution, binds single- and double-stranded DNA molecules, undergoes autophosphorylation *in vitro*, possesses a weak ATPase activity, and strongly inhibits the SsoMCM DNA helicase activity (13). In addition, we demonstrated that SsoCdc6-2 has a modular organization since a C-terminally deleted form of the protein (named ΔC) retains the ability to bind and hydrolyze ATP, whereas the C-terminal Winged Helix (WH) domain, produced separately in recombinant form (named ΔN), is able to bind single- and double-stranded DNA and to inhibit the SsoMCM DNA helicase activity (14). Herein we report evidence that SsoCdc6-2 physically interacts with SsoMCM and promotes its loading onto DNA. The ATP and DNA binding activities of SsoCdc6-2 are not required for this function.

EXPERIMENTAL PROCEDURES

Proteins—SsoMCM, wild type and KA mutant SsoCdc6-2, and SsoCdc6-2 ΔC were purified as described (12–14). The production of SsoMCM Δ268 will be published elsewhere.[2]

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[1] The abbreviations used are: Sso, *Sulfolobus solfataricus*; WH, Winged Helix; MCM, mini-chromosome maintenance.

[2] M. De Felice, B. Pucci, and F. M. Pisani, manuscript in preparation.

DNA Substrates—Oligonucleotides were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham Biosciences). To prepare the double-stranded substrates the labeled oligonucleotide was annealed to a 3-fold molar excess of a cold complementary strand. The substrate containing a bubble made from a duplex surrounding poly(dT) (Bub-20T) had the following sequence: 5′-TCTACCTGGACGACCGGG-$(T)_{20}$GGGCCAGCAGGTCCATCA-3′. Forked (Fork) and tailed duplex substrates were constructed by annealing the appropriate combinations of the following oligonucleotides: 5′-GCTCGGTACCCGGGGATCCT-CTAGA$(T)_n$-3′ and 5′-$(T)_n$TCT-AGAGGATCCCCGGGTACCGAGC-3′ (where $n = 0$ or 20). When $n = 0$ for both substrates, a duplex of 25 bp was produced. The oligonucleotide (5′-CCCAGTCACGACGTTGTAAA-ACGACGGCCAGTGCGAGGCGCGCGAAGACCG-3′) was used as a single-stranded ligand in DNA band shift assays.

DNA Band Shift Assays—For each substrate, 10-μl mixtures were prepared that contained 200 fmol of ^{32}P-labeled DNA in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5 mM $MgCl_2$, 0.7 mM 2-mercaptoethanol and the indicated amounts of SsoCdc6-2 and/or SsoMCM. Following incubation for 10–15 min at room temperature, complexes were separated by electrophoresis through 5% polyacrylamide/bisacrylamide gels (37.5:1) in 0.5× Tris borate/EDTA. Gels were dried down and quantified on a Storm PhosphorImager using ImageQuant software (Amersham Biosciences).

Immunoprecipitation Experiments—Mixtures (volume: 10 μl) were prepared that contained wild type (or ΔC) SsoCdc6-2 (1 μg) and/or full-size (or Δ268) SsoMCM (0.2 μg) and 200 fmol of DNA Bub-20T (where indicated) in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5 mM $MgCl_2$, 0.7 mM 2-mercaptoethanol. These mixtures were incubated at room temperature for 10–15 min, then 20 μl of Dynabeads M-280 sheep anti-rabbit IgG (Dynal) conjugated with anti-SsoCdc6-2 antibodies were added, and incubation was continued at room temperature for 2 h with gentle shaking. After abundant washing with buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10% glycerol, 1% Triton X-100), the beads were resuspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 100 mM 2-mercaptoethanol, 0.6% SDS, 0.01% bromphenol blue). After boiling, the samples were analyzed by Western blot using anti-SsoCdc6-2 or anti-SsoMCM antibodies.

RESULTS

DNA Binding Activity of SsoCdc6-2 and SsoMCM—The ability of SsoCdc6-2 to bind DNA in a structure-dependent manner was assayed using a standard electrophoretic mobility shift assay on a variety of synthetic oligonucleotides: molecules containing a bubble of 20 T residues (Bub-20T), flayed duplexes with tails of 20 T residues (Fork), 3′- or 5′-tailed duplexes (3′-Tail and 5′-Tail), blunt double-stranded DNA molecules, and single-stranded DNA oligonucleotides. As shown in Fig. 1, SsoCdc6-2 binds preferentially to DNA molecules that contain a bubble, a fork, or a tail and produces various shifted bands, the intensity of which was dependent upon the protein concentration. DNA molecules containing bubbles of 60 residues (with the sequence $(TTTTTA)_{10}$) or 10 T residues were also used as ligands in band shift experiments, and both were found to be bound with similar affinity with respect to the Bub-20T.[2] On the other hand, single-stranded oligonucleotides (see Fig. 1) or blunt duplexes[2] were bound with noticeably lower affinity.

Similar band shift experiments were carried out to analyze whether SsoMCM bound DNA with any structural specificity. We found that SsoMCM bound all the DNA ligands tested with higher affinity with respect to SsoCdc6-2. As shown in Fig. 2, at low protein concentration binding of SsoMCM to bubble- or fork-containing ligands produced various retarded bands (see gels in Fig. 2, *lanes 2–6*). At higher protein concentrations, the faster migrating bands disappeared, and only a lower mobility complex was observed (see gels in Fig. 2, *lanes 7* and *8*). Because SsoMCM hexamers are not stable and dissociate upon dilution (12), it is likely that the faster migrating bands are produced by binding to DNA of these various SsoMCM subassemblies, whereas the slower migrating complex found at the higher protein concentrations could contain one or possibly two SsoMCM hexamers.

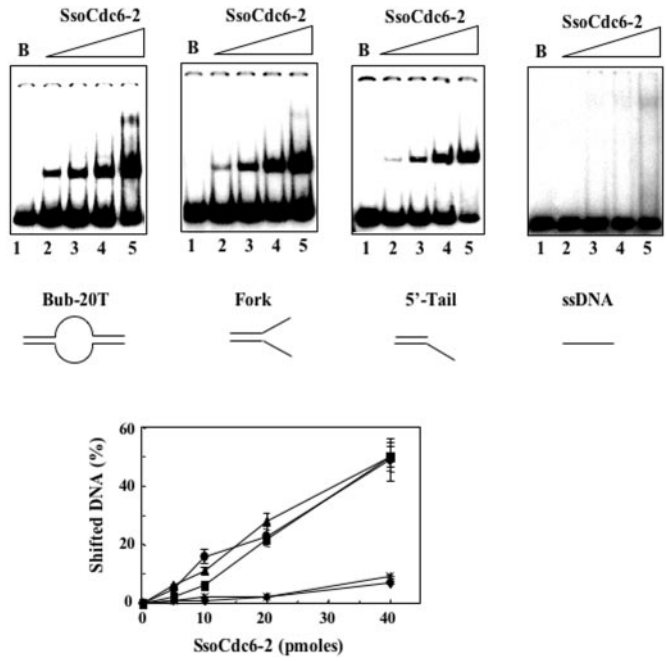

FIG. 1. **DNA binding activity of SsoCdc6-2 on various DNA molecules.** DNA band shift assays were carried out on the DNA molecules schematically indicated using increasing amounts of SsoCdc6-2 (5, 10, 20, and 40 pmol, *lanes 2–5* in each gel) as described under "Experimental Procedures." The *lanes* marked with *B* were loaded with control samples without protein. A *plot* of the shifted DNA *versus* the amount of protein used is shown (*lower panel*). Data reported are mean values of at least three independent experiments. Symbols used are: *circles* (Bubble-20T), *squares* (Fork), *triangles* (5′Tail), *diamonds* (No-Tail), and *asterisks* (single-stranded DNA).

SsoCdc6-2 Stimulates Binding of SsoMCM to Bubble- or Fork-containing DNA Molecules—We decided to analyze the mutual effect of SsoCdc6-2 and SsoMCM on the binding to oligonucleotides containing either a bubble or a fork structure. When SsoCdc6-2 was incubated alone with the bubble-containing DNA molecules it produced a main retarded band in electrophoretic mobility shift assays (Complex I in Fig. 3*A*, *lane 2*). On the other hand, SsoMCM (0.5–1.0 pmol) incubated alone with the bubble-containing DNA molecules formed a single slow migrating complex (Complex II in Fig. 3*A*, *lanes 5* and *6*). When SsoCdc6-2 and SsoMCM were mixed together in the above experimental conditions, we observed a gradual reduction of Complex I (Fig. 3*B*) together with an increase of Complex II. In a subsequent set of band shift assays the amount of SsoMCM was kept constant at a value that produced only a minimal amount of Complex II (0.1 pmol, see Fig. 3*C*, *lane 5*), whereas the amount of SsoCdc6-2 was increased from 0 to 8 pmol, a range of values that produced a low level of DNA band shift (Fig. 3*C*, *lanes 2–4*). In these conditions we found that Complex II was increased with respect to the experiments in which SsoMCM was incubated alone, and this increase was proportional to the concentration of SsoCdc6-2 used (Fig. 3*C*, *lanes 6–8*). Complex II formation was increased by about 50-fold when 16 pmol of SsoCdc6-2 were used (see *plot* in Fig. 3*D*). These results indicate that SsoCdc6-2 promotes SsoMCM binding to bubble-containing oligonucleotides. This effect was not significantly influenced by the addition of ATP (or ADP) and was also observed on the fork-containing DNA molecules although to a lesser extent (Fig. 3*D*). Furthermore, it was not dependent on the order of addition of the two proteins to the mixtures containing the DNA molecules. A similar effect was not observed in control experiments where, instead of SsoCdc6-2, we used equal amounts of various proteins including a single-stranded DNA-binding protein (SsoSSB) (17) or a

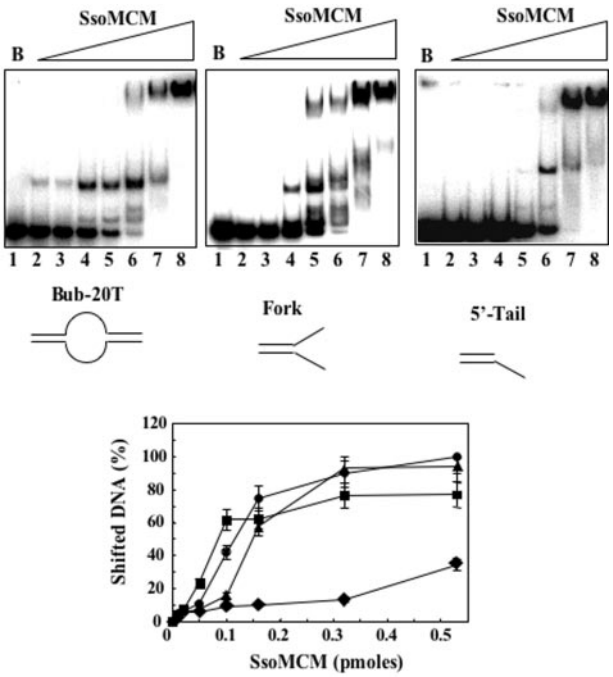

FIG. 2. **DNA binding activity of SsoMCM on various DNA molecules.** DNA band shift assays were carried out on the DNA molecules schematically indicated using increasing amounts of SsoMCM (0.01, 0.02, 0.05, 0.1, 0.16, 0.32, and 0.53 pmol, *lanes 2–8* in each gel) as described under "Experimental Procedures." The *lanes* marked with *B* were loaded with control samples without protein. A *plot* is shown of the shifted DNA *versus* the amount of protein used (*lower panel*). Data reported are mean values of at least three independent experiments. Symbols used are the same as in Fig. 1.

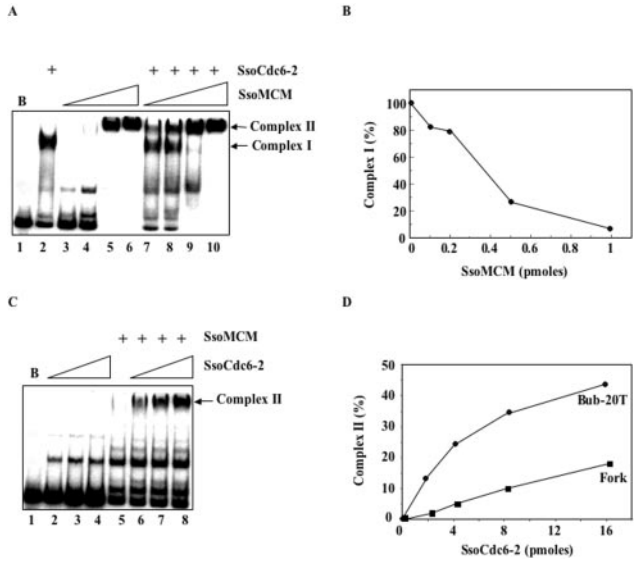

FIG. 3. **Effect of SsoCdc6-2 on the SsoMCM DNA binding activity.** *A*, DNA band shift assays were carried out on the bubble-containing DNA molecules using increasing amounts of SsoMCM (0.1, 0.2, 0.5, and 1 pmol) in the absence (*lanes 3–6*) or in the presence (*lanes 7–10*) of SsoCdc6-2 (45 pmol). *B*, the Complex I formed is plotted *versus* the amount of SsoMCM present in each assay. *C*, DNA band shift assays were carried out on the bubble-containing DNA molecules using increasing amounts of SsoCdc6-2 (2, 4, and 8 pmol) in the absence (*lanes 2–4*) or in the presence (*lanes 6–8*) of SsoMCM (0.1 pmol). *D*, the percentage of probe shifted as the Complex II in each *lane* is plotted *versus* the amount of SsoCdc6-2. In all cases, results of typical experiments are the same reported.

family B DNA polymerase (SsoDNA pol B1) (18) from *S. solfataricus*, and a carboxylesterase from the bacterium *Alycicloba-cillus acidocaldarius* (19) (data not shown).

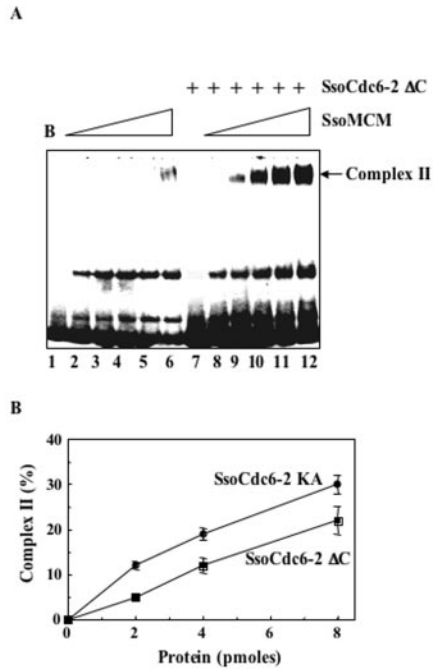

FIG. 4. **Effect of SsoCdc6-2 ΔC and KA on the SsoMCM DNA binding activity.** *A*, DNA band shift assays were carried out on the bubble-containing DNA molecules using increasing amounts of SsoMCM (0.1, 0.15, 0.2, 0.3, and 0.4 pmol) in the absence (*lanes 2–6*) or in the presence (*lanes 8–12*) of SsoCdc6-2 ΔC (28 pmol) as described under "Experimental Procedures." *B*, the percentage of probe shifted as the Complex II is plotted *versus* the amount of SsoCdc6-2 ΔC (or SsoCdc6-2 KA) used in DNA band shift experiments where 0.1 pmol of SsoMCM were present. Data are mean values of at least three independent experiments.

Loading of SsoMCM onto DNA Does Not Require the SsoCdc6-2 ATP and DNA Binding Activities—To further investigate the mechanisms by which SsoCdc6-2 stimulates binding of SsoMCM to DNA, we analyzed the loading capability of a C-terminally truncated form of SsoCdc6-2, referred to as SsoCdc6-2 ΔC. The ΔC protein contains an intact nucleotide-binding Rossmann fold but lacks the last C-terminal 103 amino acid residues that are likely to fold into a DNA-binding WH domain. We recently reported that this protein retains the ability to bind and hydrolyze ATP, but it is completely devoid of the DNA binding function (14) (see Fig. 4*A*, *lane 7*). We found that the SsoCdc6-2 ΔC protein stimulates the SsoMCM binding to DNA with an efficiency comparable with the full-size protein (see Fig. 4*A*, *lanes 8–12*). These results indicate that the SsoCdc6-2 DNA binding activity is not required to stimulate binding of SsoMCM to DNA.

In addition, we studied the effect of a SsoCdc6-2 Walker A mutant (referred to as SsoCdc6-2 KA) on the SsoMCM interaction with a bubble-containing DNA ligand by band shift assays. The SsoCdc6-2 KA protein carries a substitution of the Walker A lysine residue 59 by alanine and was shown to be completely unable to bind and hydrolyze ATP (13). We found that the SsoCdc6-2 KA mutant promoted SsoMCM binding to DNA with an efficiency similar to the wild type protein (Fig. 4*B*), indicating that integrity of the Walker A motif is not required for SsoMCM loading onto DNA.

SsoCdc6-2 and SsoMCM Physically Interact—The stimulation of the SsoMCM DNA binding activity by SsoCdc6-2 suggested that the two proteins could physically interact with one another. In a previous report we demonstrated by a gel filtration analysis that SsoMCM and SsoCdc6-2 are able to associate, but the protein complexes formed were unstable (13). In this study a physical interaction between the two proteins was

tested by immunoprecipitation experiments using Dynabeads that were conjugated with antibodies raised against the homogeneous SsoCdc6-2. As shown in Fig. 5, SsoCdc6-2 is able to associate with SsoMCM either in the absence or presence of bubble-containing DNA molecules. Likewise, the SsoCdc6-2 ΔC protein was able to physically interact with SsoMCM, indicating that its C-terminal WH domain is not necessary to establish this association.

To further investigate the physical/functional interaction between SsoCdc6-2 and SsoMCM we carried out electrophoretic mobility shift assays using a truncated form of SsoMCM, named SsoMCM Δ268. This protein lacks the first N-terminal 268 amino acid residues, including the zinc-binding motif and a cluster of basic residues that are thought to be responsible for the DNA binding function (20). We have recently found that SsoMCM Δ268 retains ATPase activity, forms homohexamers in solution but is completely unable to bind to DNA.[3] When a fixed amount of SsoMCM Δ268 (2 pmol) was mixed with SsoCdc6-2 (amounts ranging from 5 to 40 pmol), we observed a gradual reduction of the shifted Complex I with respect to control experiments where SsoMCM Δ268 was omitted (see Fig. 6*A*). A likely interpretation of these findings is that SsoMCM Δ268 interacts with SsoCdc6-2, and this interaction causes a reduction of the SsoCdc6-2 DNA binding affinity. This hypothesis was confirmed by the results of immunoprecipitation experiments carried out on mixtures of the two proteins using Dynabeads that were conjugated with antibodies raised against SsoCdc6-2 (Fig. 6*B*). These findings also indicate that the first N-terminal 268 amino acid residues of the SsoMCM polypeptide chain are not necessary for the physical interaction with SsoCdc6-2.

[3] B. Pucci, M. De Felice, and F. M. Pisani, manuscript in preparation.

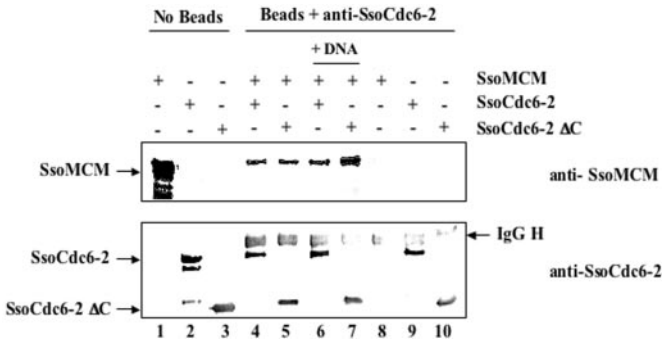

FIG. 5. **Physical interaction between SsoCdc6-2 and SsoMCM.** Immunoprecipitation experiments were carried out using Dynabeads conjugated with anti-SsoCdc6-2 antibodies as described under "Experimental Procedures." Samples were analyzed by Western blot, using antisera directed against SsoMCM and SsoCdc6-2 and anti-rabbit alkaline phosphatase-conjugated secondary antibodies. A conventional colorimetric reaction was carried out to detect bound secondary antibodies.

DISCUSSION

All sequenced archaeal genomes were found to contain genes putatively coding for Orc1/Cdc6 homologs, the only exception being *Methanococcus jannaschii*. However, whereas certain species, such as those of the *Pyrococcus* genus and *Pyrobaculum aerophilum*, have a single Orc1/Cdc6-like protein, several other archaea have multiple Orc1/Cdc6 homologs. It is quite likely that in the species having a single Orc1/Cdc6 homolog this factor may function either as the replication initiator or as the replicative DNA helicase loader. On the other hand, in species with multiple Orc1/Cdc6 factors they may play different roles in the establishment of active replication forks. The hyperthermophilic crenarchaea *S. solfataricus* possesses three Orc1/Cdc6-like initiation factors. The biological function of these proteins has not yet been clearly defined. In the current study we demonstrate that SsoCdc6-2 binds DNA molecules that mimic early replication intermediates in a sequence-independent manner and stimulates binding of the SsoMCM DNA helicase to these DNA ligands. In a previous study we showed that the SsoMCM homohexamers are not stable but dissociate in solution into various subhexameric complexes (12). We propose that the SsoMCM loading process is of the ring assembly type, being likely to proceed through the recruitment of the helicase monomers and their oligomerization around DNA. Our analysis indicates that the SsoCdc6-2 DNA-binding WH domain is not necessary to establish an association with SsoMCM and to promote its loading onto DNA. In fact, we showed that these functions are also carried out by a C-terminally truncated form of SsoCdc6-2, which only contains an AAA^+ (ATPases with associated cellular activities) module (14). Our current working hypothesis is that the SsoCdc6-2 ATPases with asso-

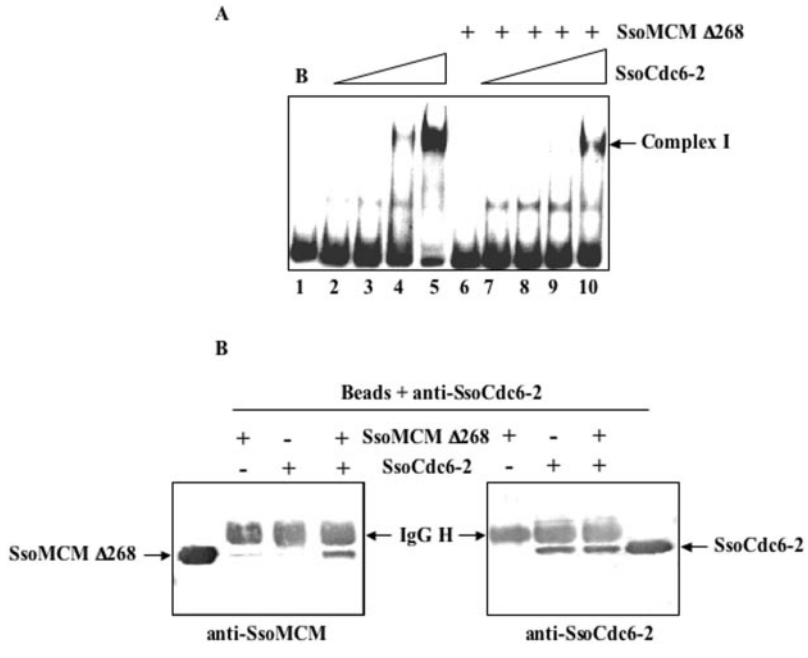

FIG. 6. **Effect of SsoMCM Δ268 on the SsoCdc6-2 DNA binding activity.** *A*, DNA band shift assays were carried out on the bubble-containing DNA molecules using increasing amounts of SsoCdc6-2 (5, 10, 20, and 40 pmol) in the absence (*lanes 2–5*) or in the presence (*lanes 7–10*) of SsoMCM Δ268 (2 pmol) as described under "Experimental Procedures." *B*, physical interaction between SsoCdc6-2 and SsoMCM Δ268 was investigated by immunoprecipitation experiments carried out using Dynabeads conjugated with anti-SsoCdc6-2 antibodies. Samples were analyzed by Western blot, using antisera directed against SsoMCM and SsoCdc6-2 and anti-rabbit alkaline-phosphatase-conjugated secondary antibodies. A conventional colorimetric reaction was carried out to detect bound secondary antibodies.

ciated cellular activities module could promote the oligomerization of the SsoMCM monomers, although an integral Walker A motif does not seem to be required for this function. On the other hand, the SsoCdc6-2 WH domain could be responsible for the recruitment of SsoMCM at the replication origins by means of its ability to bind DNA in a sequence-specific manner (15). However, SsoCdc6-2 could also play a role in loading SsoMCM at stalled replication forks, because it binds and promotes binding of this hexameric DNA helicase to DNA in a structure-dependent fashion.

It was recently shown that the MCM-like complex and one of the two Cdc6 homologs of the Archaea *Archeoglobus fulgidus* bind with higher affinity DNA molecules containing a single-stranded bubble. However, in contrast with our results, the *A. fulgidus* Cdc6 factor was found to displace MCM hexamers bound to these DNA molecules (21). It is interesting to note that in a recent sequence analysis of the Cdc6-like proteins from various archaeal species, SsoCdc6-2 and the above *A. fulgidus* Cdc6 factor were reported to belong to different phylogenetic clades, thus being likely that they may play different roles at the replication origins (22). However, the two other *S. solfataricus* Cdc6 factors have been recently characterized by us and discovered to promote binding of SsoMCM to bubble- or fork-containing DNA molecules with an efficiency similar to SsoCdc6-2.[2] In addition, we have found that SsoCdc6-1 and -2 inhibit the SsoMCM DNA unwinding function *in vitro*, as reported previously in the case of SsoCdc6-2 (13). Thus, we postulate that SsoMCM is loaded onto DNA at the replication origins in an inactive form and released as an active DNA helicase at the onset of mitosis by the action of other replication factors. Our ongoing biochemical analysis of the interplay among the various *S. solfataricus* initiation factors will help in unraveling the mechanisms of replisome assembly in this species and may also provide important clues toward understanding the molecular mechanisms by which replicative DNA helicases are recruited and loaded onto DNA during chromosomal DNA replication in the more complex eukaryotic organisms.

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