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Three eukaryote-like Orc1/Cdc6 proteins functionally interact and mutually regulate their activities of binding to the replication origin in the hyperthermophilic archaeon *Sulfolobus solfataricus* P2

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Abstract

The crenarchaeon *Sulfolobus solfataricus* has the potential to be a powerful model system to understand the central mechanism of eukaryotic DNA replication because it contains three active origins of replication and three eukaryote-like Orc1/Cdc6 proteins. However, it is not known whether these SsoCdc6 proteins can functionally interact and collectively contribute to DNA replication initiation. In the current work, we found that SsoCdc6-1 stimulates DNA-binding activities of SsoCdc6-3. In contrast, SsoCdc6-3 inhibits those of both SsoCdc6-1 and SsoCdc6-2. These regulatory functions are differentially affected by the C-terminal domains of these SsoCdc6 proteins. These data, in conjunction with studies on physical interactions between these replication initiators by bacterial two-hybrid and pull-down/Western blot assays, lead us to propose the possibility that multiple SsoCdc6 proteins might coordinately regulate DNA replication in the archaeon species. This is the first report on the functional interaction among the archaeal multiple Cdc6 proteins to regulate DNA replication.

Keywords: Archaea; Orc1/Cdc6; DNA replication; *Sulfolobus solfataricus*

DNA replication, a very tightly regulated process, is essential to all three domains of life: Bacteria, Eukarya, and Archaea. Many sequenced archaeon genomes encode the DNA replication proteins, which more closely resemble the proteins present in eukaryotes compared to bacteria [1–3]. In recent years, it has become apparent that the archaeal replication machinery represents a core version of that in eukaryotes. Therefore, archaea is one of the best model organisms for studying DNA replication and will provide relevant details on what is happening in eukaryotes [4–6].

Proper recognition of replication origins is crucial for origin firing and for further assembling a functional replisome. During the initiation stage, the origin of replication is recognized by a protein complex, leading to the assembly of the helicase onto DNA [7]. Replication initiation in bacteria is typically controlled by a single protein, DnaA, which binds to repeated sequence elements known as DnaA boxes that lie within a single chromosomal origin, *oriC* [8]. Once the melted origin–DnaA complex has formed, the replicative helicase DnaB can be loaded by DnaA together with DnaC [9,10]. Interestingly, DnaC, like DnaA, is also a member of the AAA^+ family of ATPases [8].

In contrast to the single and clearly defined sites of bacterial replication, eukaryotic DNA replication involves the ordered assembly of a number of replication factors at multiple origin sites that are bound by a six-subunit (Orc1–6) complex called the origin recognition complex (ORC) [8,11]. DNA replication is initiated by the formation of the pre-replicative complex (pre-RC) and the regulation of pre-RC is a key element of the mechanisms coordinating DNA replication with the cell cycle [11]. One candidate to increase the DNA-binding specificity of ORC to origin sequences is the Cdc6 which interacts directly with Orc1

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[11,12]. Orc1 and Cdc6 are closely related and presumably from a common ancestor [8].

Archaeaon species also have some of the pre-RC components, including a MCM-like helicase and one or more copies of Orc1/Cdc6 proteins that are highly sequence related to both Orc1 and Cdc6 [13]. Within the archaeal homologs of eukaryotic replication proteins, the most likely candidates for initiator proteins are the Orc1/Cdc6 proteins which can specifically bind to the replication origins either *in vivo* or *in vitro* [14–16]. The role of the Orc1/Cdc6 protein appears to be both origin recognition and MCM loading. The three-dimensional structure of archaeal Orc1/Cdc6 protein has been shown to contain an AAA^+ domain and a winged helix (WH) domain, both of which are predicted to be conserved in the eukaryotic Orc1 and Cdc6/Cdc18 [17,18].

Multiple origins indeed exist in the archaeon species of *Sulfolobus solfataricus*, *Sulfolobus acidocaldarius*, and *Aeropyrum pernix* [16,19,20]. Interestingly, many archaeal origin sequences physically map to sites adjacent to the genes encoding the Orc1/Cdc6 protein [3]. Three Orc1/Cdc6 proteins have been identified and each origin is preferentially bound by the distinct subset of Cdc6 proteins in *S. solfataricus*. The three Cdc6 proteins bind specially to repeat motifs found at the *oriC1* and *oriC2* origin [16]. In contrast, all three Cdc6 proteins could also bind to the *oriC3* origin, these interactions were not centered on the new repeats [21]. In addition, the study also identified sequence motifs within the origin sequences specially bound by the Cdc6 proteins and similar sequence motifs have been identified in other archaeal origins [16]. These results suggest that the multiple Cdc6 proteins may oligomerize at the origin to form a structure similar to the multiple DnaA proteins that form at the *Escherichia coli oriC*. However, more biochemical studies are needed to investigate how the Orc1/Cdc6 protein functions to recognize the origins of DNA replication.

The functional interactions between these Orc1/Cdc6 proteins have not yet been reported in the archaeon species containing multiple replication origins and Orc1/Cdc6-like proteins which provide a powerful tool to address the mechanism of origin selection and cell cycle control of replication, and that are still not completely understood in eukaryotes [14]. Our recent studies showed that three SsoCdc6s had the different effects on their interactions with SsoMCM [22]. In order to further investigate the defined function of three SsoCdc6s during the early events of DNA replication, here we addressed the physical and functional interactions between three SsoCdc6 proteins on the duplex DNA derived from the *S. solfataricus* origin C2.

Materials and methods

DNA and oligonucleotides. All DNA and oligonucleotides were synthesized by Invitrogen. Oligonucleotides were labeled, respectively, using T4 polynucleotide kinase (TaKaRa) and [γ-^{32}P]ATP (Beijing FuRui company), purified using micro HiTrap^{TM} Desalting chromatography columns (Amersham Biosciences). The blunt and forked duplex DNA substrates were constructed, respectively, by mixing the labeled oligonucleotides with the 3-fold molar excess of their cold complementary strands. Structures and sequences of DNA substrates used are described in Figs. 1A and 2A. The sequence-specific forked DNA was designed to mimic early replication intermediates.

Cloning and purification of SsoCdc6 proteins. Prokaryotic vectors expressing SsoCdc6 proteins and untagged proteins for pull-down assay were constructed as described previously [22]. *E. coli* BL21 CodonPlus (DE3)-RIL cells (Novagen) were used as the host strain to express and purify *S. solfataricus* P2 SsoCdc6 and Cdc6ΔC proteins as described in related literature [23,24] and our published procedures previously [22]. Purified proteins were greater than 99% purity as determined by SDS–PAGE and subsequent staining by Coomassie blue. Protein concentrations were determined by spectrophotometric absorbance at 260 nm according to Gill and Hippel [25].

Electrophoretic mobility shift assays. The binding of SsoCdc6 proteins to duplex DNA was performed on the blunt and forked DNA (Figs. 1A and 2A) using a modification of Electrophoretic Mobility Shift Assay (EMSA) as described in related literature [23,24] and our published procedures previously [22,26,27]. The reactions (10 μl) for measuring the mobility shift contained 200 fmol ^{32}P-labeled duplex DNA and various indicated amounts of SsoCdc6 protein concentrations diluted in buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5 mM $MgCl_2$, and 0.7 mM 2-mercaptoethanol. Reaction mixtures were incubated at room temperature for 20 min before loading on 5% polyacrylamide/bis (37.5:1) gels in 0.5× TBE. Gels were dried and exposed to a FujiX PhosphorImager plate, and the fraction of DNA bound by archaeal proteins was measured using ImageQuant software.

Bacterial two-hybrid analysis. BacterioMatch II Two-Hybrid System Library Construction Kit (Stratagene) was used to detect protein–protein interactions between SsoCdc6 proteins. Bacterial two-hybrid analysis was carried out according to the procedure offered by the commercial kit. pBT and pTRG vectors containing archaeal genes of SsoCdc6/Cdc6ΔC were generated. All the primers used for PCR amplification are described in Supplementary Table 1. Positive growth co-transformants were selected on the Selective Screening Medium plate containing 5 mM 3-AT (Stratagene), 8 μg/ml streptomycin, 15 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin. Co-transformant containing pBT-LGF2 and pTRG-Gal11^P (Stratagene) was used as positive control for an expected growth on the Selective Screening Medium and co-transformant containing empty vector pBT and pTRG was used as negative control.

Pull-down and Western blot assays. Pull-down assays were carried out as described previously [22]. The untagged-SsoCdc6 (100 μg) was pre-incubated with 50% Ni–NTA agarose (Qiagen) in buffer A (25 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 100 mM NaCl, 30% glycerol, and 10 mM imidazole) in a volume of 200 μl at 4 °C for 1 h and centrifuged. The supernatant was used for the pull-down assays. One hundred micrograms of untagged-SsoCdc6 protein was mixed with another His-tagged SsoCdc6 protein (100 μg) as indicated in Fig. 4 into a 500 μl incubation buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.5 mM $MgCl_2$ and incubated at 25 °C for 10 min, then co-incubated at 4 °C for 1 h with Ni–NTA agarose pre-equilibrated with buffer A. The beads were then washed twice with 1 ml buffer A containing 20 mM imidazole and centrifuged at 4500 rpm for 1 min. Proteins bound to the beads were eluted with 100 μl elution buffer A containing 400 mM imidazole. The elutes were then analyzed on 10% SDS–PAGE and further analyzed by Western blot using anti-SsoCdc6 antibodies.

Results

Origin sequence-specific DNA substrates designed for studying the interactions of three SsoCdc6 proteins

It is known that *Sulfolobus* species have three replication origins on the circular chromosome and its genome has

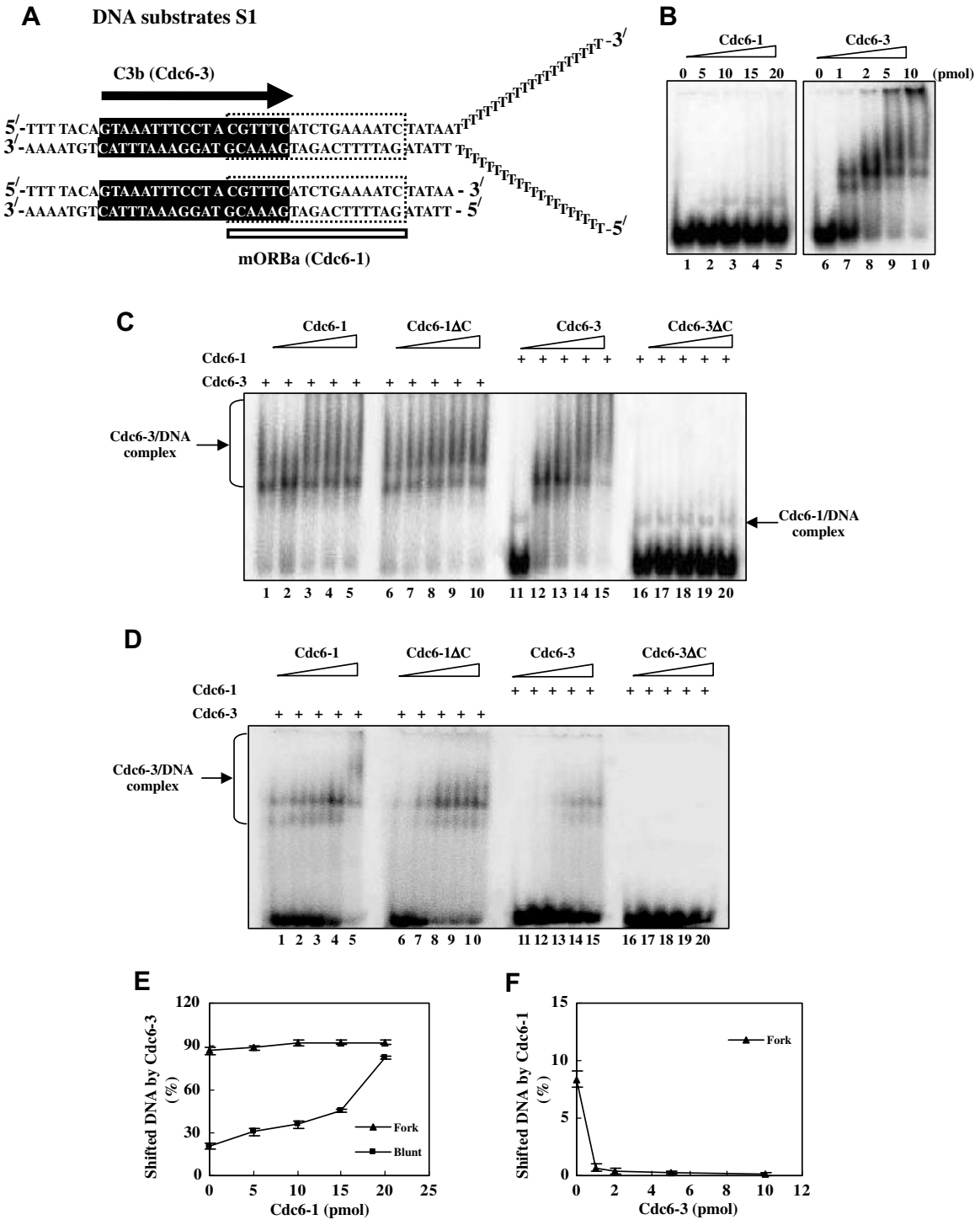

Fig. 1. Mutual effects between SsoCdc6-1 and SsoCdc6-3 on their binding to DNA substrates S1. The EMSAs were carried out and the amount of radioactivity in each specific band was measured as described under Materials and methods. (A) Sequences and structures of the DNA substrates S1 used for EMSAs. DNA substrates S1 contains two partially overlapped binding sites for SsoCdc6-1 and SsoCdc6-3 and was designed based on the sequences and structures of the native *S. solfataricus* P2 origin C2 (16). (B) An increasing amount of single SsoCdc6-1 (0, 5, 10, 15, and 20 pmol) or SsoCdc6-3 (0, 1, 2, 5, and 10 pmol) protein bound to substrate S1 to produce specific protein/DNA complex. (C) The effects of an increasing amount of SsoCdc6-1 or SsoCdc6-1ΔC (0, 5, 10, 15, and 20 pmol) on the DNA-binding activities of the fixed amount of SsoCdc6-3 (3 pmol) and those of an increasing amount of SsoCdc6-3 or SsoCdc6-3ΔC (0, 1, 2, 5, and 10 pmol) on the DNA-binding activities of the fixed amount of SsoCdc6-1 (20 pmol) to the forked DNA substrates S1. (D) The effects of an increasing amount of SsoCdc6-1 or SsoCdc6-1ΔC (0, 5, 10, 15, and 20 pmol) on the DNA-binding activities of the fixed amount of SsoCdc6-3 (3 pmol) and those of an increasing amount of SsoCdc6-3 or SsoCdc6-3ΔC (0, 1, 2, 5, and 10 pmol) on the DNA-binding activities of the fixed amount of SsoCdc6-1 (20 pmol) to the blunt DNA substrates S1. A plot of a varied amount of SsoCdc6-1 or SsoCdc6-3 versus amounts of the shifted DNA (%) by another fixed amount of SsoCdc6-3 (3 pmol) (E) or SsoCdc6-1 (20 pmol) (F), respectively. Data reported are mean values of at least three independent experiments.

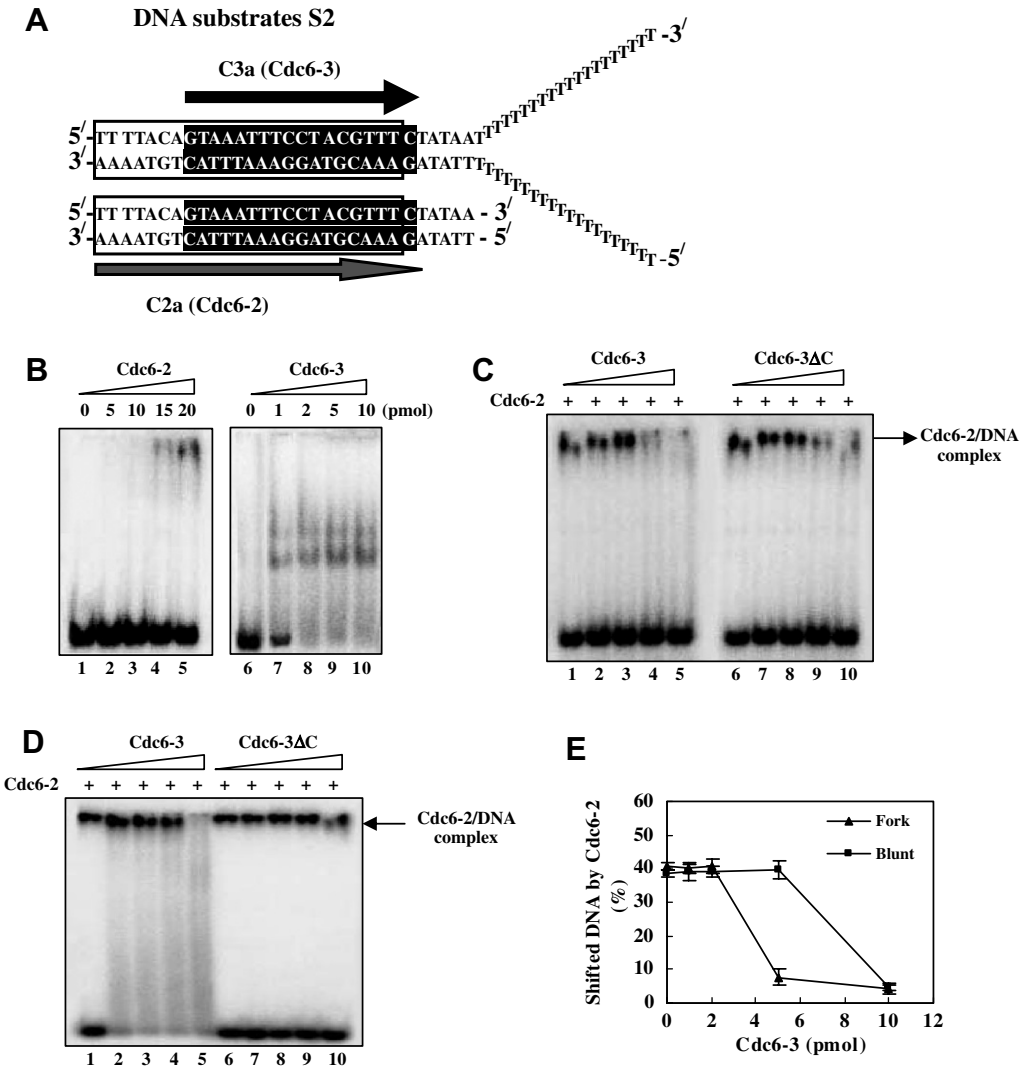

Fig. 2. Mutual effects between SsoCdc6-2 and SsoCdc6-3 on their binding to DNA substrates S2. The EMSAs were carried out and the amount of radioactivity in each specific band was measured as described under Materials and methods. (A) Sequences and structures of the DNA substrates S2 used for EMSAs. DNA substrates S2 designed based on the sequences and structures of the native *S. solfataricus* P2 origin C2 (16) contains two sequence boxes, in which position of the Cdc6-3-binding sites encompassed within that of the SsoCdc6-2 protein. (B) An increasing amount of single SsoCdc6-2 (0, 5, 10, 15, and 20 pmol) or SsoCdc6-3 (0, 1, 2, 5, and 10 pmol) protein bound to substrate S2 to produce specific protein/DNA complex. (C) The effects of an increasing amount of SsoCdc6-3 or SsoCdc6-3ΔC (0, 1, 2, 5, and 10 pmol) on the DNA-binding activities of the fixed amount of SsoCdc6-2 (20 pmol) to the forked DNA substrates S2. (D) The effects of an increasing amount of SsoCdc6-3 or SsoCdc6-3ΔC (0, 1, 2, 5, and 10 pmol) on the DNA-binding activities of the fixed amount of SsoCdc6-2 (20 pmol) to the blunt DNA substrates S2. (E) A plot of a varied amount of SsoCdc6-3 (0, 1, 2, 5, and 10 pmol) versus the amounts of the shifted DNA (%) by the fixed amount of SsoCdc6-2 (20 pmol). Data reported are mean values of at least three independent experiments.

three genes encoding eukaryotic-like Orc1/Cdc6. Moreover, a eukaryotic type cell cycle was reported [16,19]. Previous studies have shown that SsoCdc6 can recognize the sequence-specific double-strand DNA motifs and have the different effects on the interactions with SsoMCM [22]. It is therefore an important question whether there are any functional interactions among the three Cdc6 proteins to regulate DNA replication. Interestingly, the positions of some binding sites for three SsoCdc6 proteins are overlapped, which suggests potential interactions of multiple SsoCdc6 proteins within the origins [16].

In order to better understand and clearly define the molecular interactions of three eukaryotic-like Orc1/Cdc6 proteins on the origins in the archaeon species, we designed two groups of forked and blunt DNA substrates based on the positions of origin binding sequence for SsoCdc6 protein within *oriC2* characterized by Robinson et al. [16]. DNA substrates S1 contains site for the binding of SsoCdc6-1 (mORBa) overlapped with that of SsoCdc6-3 (C3a) (Fig. 1A). DNA substrates S2 contains the SsoCdc6-3-binding site (C3a) which is encompassed within the SsoCdc6-2-binding site (C2a) (Fig. 2A).

Using these purified SsoCdc6 proteins and a standard electrophoretic mobility shift assay (EMSA), we examined the specific protein/DNA complex species for each

SsoCdc6 protein and the functional interactions between three SsoCdc6 proteins on the three groups of DNA substrates. BSA was used as a negative control for protein and no BSA/DNA complex was observed (data not shown).

SsoCdc6-1 stimulated DNA binding of SsoCdc6-3, but SsoCdc6-3 inhibited the binding of SsoCdc6-1 to the forked and blunt DNA substrate S1

When 0–20 pmol SsoCdc6-1 protein or 0–10 pmol SsoCdc6-3 was incubated alone with a fixed concentration of the forked substrate S1 (Fig. 1A), we observed their specific DNA/protein shift band. As show in Fig. 1B, binding of SsoCdc6-1 to substrate S1 produced a slow migrating band (Fig. 1B, lanes 1–5). On the other hand, binding of SsoCdc6-3 to substrate S1 produced various retarded bands (Fig. 1B, lanes 6–10).

We further analyzed the mutual effects of SsoCdc6-1 and SsoCdc6-3 on their activities of binding to substrate S1. When an increasing amount of SsoCdc6-1 and 3 pmol SsoCdc6-3 was mixed together, a slightly increasing SsoCdc6-3/DNA complex was observed (Fig. 1C, lanes 1–5). SsoCdc6-1ΔC also kept the similar effect on the formation of protein/DNA complex as its wild-type protein (Fig. 1C, lanes 6–10). Interestingly, more strikingly stimulating effects of both SsoCdc6-1 and SsoCdc6-1ΔC, which lost the activities of binding on the blunt DNA substrate S2 (data not shown), were observed on blunt DNA substrate than on the forked substrate (Fig. 1D, lanes 1–10 and E).

On the other hand, the DNA-binding activity of SsoCdc6-1 was inhibited by SsoCdc6-3 when an increasing amount of SsoCdc6-3 was mixed together with 20 pmol SsoCdc6-1(Fig. 1C, lanes 11–15 and 1F). SsoCdc6-3ΔC, a C-terminally deleted form of the SsoCdc6-3 protein, lost the inhibitive effect on the DNA-binding activity of SsoCdc6-1 (Fig. 1C, lanes 16–20).

Thus, SsoCdc6-1 and SsoCdc6-1ΔC stimulated DNA binding of SsoCdc6-3. SsoCdc6-3 competitively inhibited

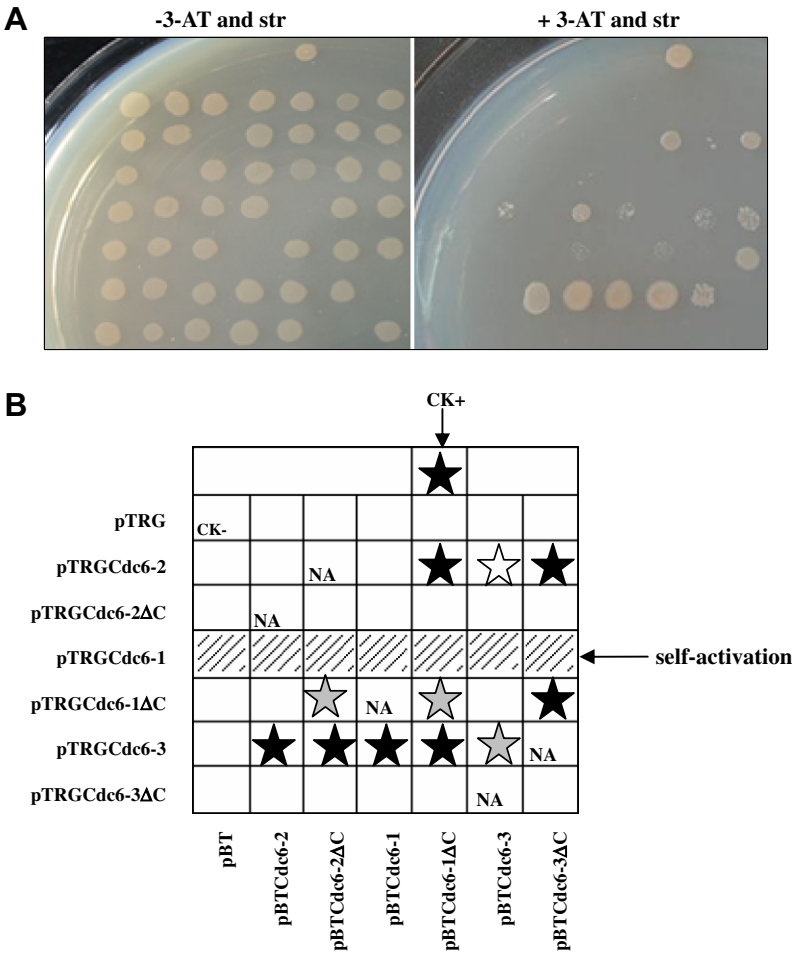

Fig. 3. Two-hybrid assays for the interactions between SsoCdc6 proteins. The BacterioMatch II two-hybrid system (Stratagene) was used to detect protein–protein interactions between SsoCdc6 proteins which were performed as described under Materials and methods. (A) Left panel, plate minus streptomycin (str) and 5 mM 3-amino-1,2,4-triazole (3-AT), Right panel, plate plus str and 5 mM 3AT. (B) An outline of the plates in A. ☆ represents positive reaction, the black one represents the stronger interaction, the gray represents the strong interaction, and the blank one represents the weak interaction. NA, no data available. CK^+, co-transformant containing pBT-LGF2 and pTRG-Gal11^P as a positive control. CK^-, co-transformant containing pBT and pTRG as a negative control.

the binding of SsoCdc6-1 to the DNA substrate S2, but SsoCdc6-3ΔC lost the inhibitive activity.

SsoCdc6-3 inhibited DNA-binding activity of SsoCdc6-2 to the forked and blunt DNA substrate S2

When an increasing single SsoCdc6 protein was incubated alone with a fixed concentration of the forked substrate S2, we observed their specific DNA/protein shift band for both SsoCdc6-2 and SsoCdc6-3 proteins (Fig. 2B). The SsoCdc6-2/DNA complex appeared to be significantly reduced when an increasing amount of SsoCdc6-3 was mixed together with 20 pmol SsoCdc6-2 on both forked (Fig. 2C, lanes 1–5) and blunt substrates (Fig. 2D, lanes 1–5). SsoCdc6-3ΔC retained a similar effect on the formation of SsoCdc6-2/DNA complex (Fig. 2C and D, lanes 6–10). However, with respect to experiments in which SsoCdc6-3 was not added in the reactions (Fig. 2B, lanes 6–10), the binding activity of SsoCdc6-3 was not observed on the forked substrates (Fig. 2C, lanes 1–5). These results demonstrate that SsoCdc6-3 and SsoCdc6-3ΔC inhibited the DNA-binding activity of SsoCdc6-2 (Fig. 2F).

Physical interactions between SsoCdc6 proteins

Functional interactions suggest a direct interaction between two of the three SsoCdc6 proteins, which prompted a further analysis of the physical interactions between a pair of proteins of three SsoCdc6 proteins using bacterial two-hybrid system. As expected in bacterial two-hybrid assays, a positive co-transformant grew up on the Selective Screening Medium, but the negative one did not grow at all. Meanwhile, we observed the SsoMCM self-interaction hopefully as the molecule forms a hexamer, and observed the interaction between SsoMCM and single-strand DNA-binding protein (SSB) (data not shown) of *S. solfataricus* shown earlier [28]. These results indicated that bacterial two-hybrid system here worked well to detect the protein–protein interactions of the hyperthermophilic archaeon. Using two-hybrid system, we observed the following protein–protein interactions: SsoCdc6-1/SsoCdc6-3, SsoCdc6-1ΔC/SsoCdc6-3, SsoCdc6-2/SsoCdc6-3, and SsoCdc6-2/SsoCdc6-3ΔC (Fig. 3).

In pull-down experiments, untagged proteins were pre-incubated with Ni–NTA agarose in order to eliminate some non-specific binding. BSA used as a negative control was not observed to pull down by three His-tagged SsoCdc6 proteins (data not shown). As shown in Fig. 4, the interactions of SsoCdc6-3/SsoCdc6-2, SsoCdc6-2/SsoCdc6-3ΔC, and SsoCdc6-3/SsoCdc6-1 were clearly detected in the absence or presence of forked DNA substrate S2.

It has been reported that SsoCdc6-3 was able to interact with SsoCdcd6-1 and SsoCdc6-2, which was tested using the Biacore system [29]. Using bacterial two-hybrid and pull-down/Western blot assays here we observed the above interactions.

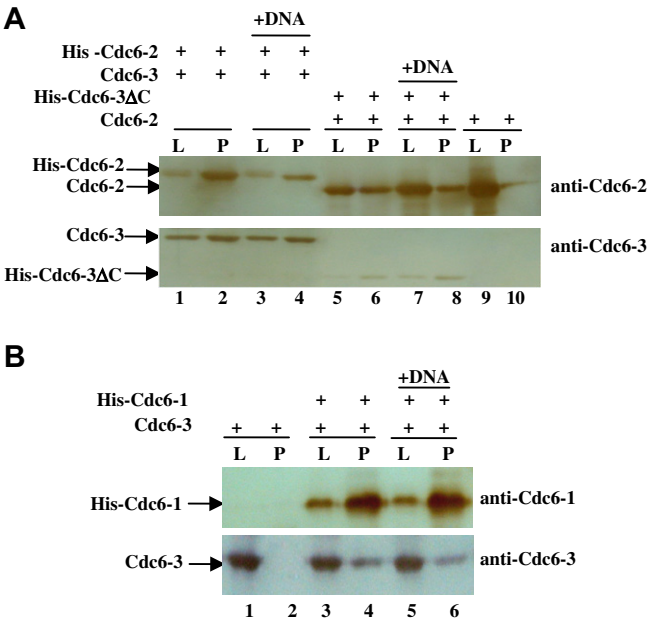

Fig. 4. Physical interactions between three SsoCdc6 proteins assayed by Protein pull-down/Western blot assays. (A) The interactions between SsoCdc6-2 and SsoCdc6-3 or SsoCdc6-3ΔC. (B) The interactions between SsoCdc6-1 and SsoCdc6-3. The effect of DNA was examined by adding S2 forked DNA or not in the reaction mixture. The proteins eluted from the Ni–NTA agarose using buffer A containing 20 mM imidazole are marked by 'L' and those using buffer A containing 400 mM imidazole are marked by 'P'. The elutes were analyzed on 10% SDS–PAGE and further analyzed by Western blot using anti-SsoCdc6 antibodies.

Discussion

As an intricate process, DNA replication requires the coordinately and tightly regulated molecular interactions of protein–protein and protein–DNA. In this study, we have characterized the physical and functional interactions of multiple SsoCdc6 proteins using two groups of DNA substrates. Three SsoCdc6 proteins were shown to stimulate or inhibit the DNA-binding activities of others. Therefore it suggests a direct interaction between two of the three SsoCdc6 proteins. Excitingly, the interactions between SsoCdc6-1ΔC and SsoCdc6-3, and SsoCdc6-2 and SsoCdc6-3ΔC were observed, respectively, using bacterial two-hybrid and pull-down/Western blot assays here. These results are perfectly consistent with the functional interactions that SsoCdc6-1ΔC retained stimulating the DNA-binding activities of SsoCdc6-3, and SsoCdc6-3ΔC maintained inhibitive effect on the DNA binding of SsoCdc6-2. Furthermore, SsoCdc6-3 was demonstrated to interact extensively with other SsoCdc6 and SsoCdc6ΔC proteins. Specially, its C-terminal deleted protein SsoCdc6-3ΔC also maintained this kind of interactions. Therefore, SsoCdc6-3 protein might have an unusual role during DNA replication initiation.

The previous reports suggest that the interactions between SsoCdc6 proteins might facilitate the assembly of a cooperative complex at the origin based on the situation of binding site of three SsoCdc6 proteins on origin [16]. Interestingly, this study has been shown that three SsoCdc6 proteins have significant differences on the binding to the DNA substrates. Three SsoCdc6/DNA complexes migrate quite differently, despite the fact that the three proteins are paralogs with sequence similarities and similar molecular weight.

Another interesting finding is that three SsoCdc6 proteins show obviously different affinities to their specific DNA substrate. SsoCdc6-3 appeared to have the highest DNA-binding activity (Figs. 1B and 2B). In contrast, SsoCdc6-1 was shown to bind DNA very weakly and even no obvious by shifted band was produced on the blunt substrate (Fig. 1D) which is consistent with a previous result [29] although a specific band can be observed clearly on the forked DNA substrates as shown in Fig. 1C. It has been found that the three Cdc6 genes show different expression profiles during the cell cycle of *S. acidocaldarius*. Whereas Cdc6-1 and Cdc6-3 proteins are expressed in G1- and S-phase cells, Cdc6-2 is present during the G2-phase [16]. It has been suggested that these proteins, collectively, might contribute to positively and negatively regulating DNA replication initiation. The dramatic difference in the expression profile and our finding on differential interactions of the three proteins raised the interesting possibility. Multiple Orc1/Cdc6 proteins may exist in many archaeal species as an integral component of pre-RC. Obviously, further investigation on the properties of three SsoCdc6 proteins remains to be done.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.125.

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