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Biochemical characterization of two Cdc6/ORC1-like proteins from the crenarchaeon *Sulfolobus solfataricus*

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Abstract The biological role of archaeal proteins, homologous to the eukaryal replication initiation factors of cell division control (Cdc6) and origin recognition complex (ORC1), has not yet been clearly established. The hyperthermophilic crenarchaeon *Sulfolobus solfataricus* (referred to as Sso) possesses three Cdc6/ORC1-like factors, which are named Sso Cdc6-1, Cdc6-2 and Cdc6-3. This study is a report on the biochemical characterization of Sso Cdc6-1 and Cdc6-3. It has been found that either Sso Cdc6-1 or Cdc6-3 behave as monomers in solutions by gel filtration analyses. Both factors are able to bind to various single-stranded and double-stranded DNA ligands, but Sso Cdc6-3 shows a higher DNA-binding affinity. It has also been observed that either Sso Cdc6-1 or Cdc6-3 inhibit the DNA unwinding activity of the *S. solfataricus* homo-hexameric mini-chromosome maintenance (MCM)-like DNA helicase (Sso MCM); although they strongly stimulate the interaction of the Sso MCM with bubble-containing synthetic oligonucleotides. The study has also showed, with surface plasmon resonance measurements, that Sso Cdc6-2 physically interacts with either Sso Cdc6-1 or Sso Cdc6-3. These findings may provide important clues needed to understand the biological role that is played by each of these three Cdc6 factors during the DNA replication initiation process in the *S. solfataricus* cells.

Keywords DNA replication · Cell division control protein 6 · Origin recognition complex · Archaea · *Sulfolobus solfataricus*

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Abbreviations *ORC*: Origin recognition complex · *Cdc*: Cell division control · *MCM*: Mini-chromosome maintenance · *LB*: Luria-bertani · *SsDNA*: Single-stranded DNA · *DsDNA*: Double-stranded DNA · *TBE*: Tris/borate/edta · Sso: *Sulfolobus solfataricus* · Mth: *Methanobacterium thermoautotrophicum* · *WH*: Winged helix

Introduction

The faithful duplication of genetic information, which is essential to all cells and DNA replication, is a very tightly regulated process. A proper recognition of replication origins is crucial to control the timing of origin unwinding and for recruiting and assembling a functional replisome. The coordination of these events prevents over-initiation and maintains an appropriate chromosomal copy number (Kornberg and Baker 1992a).

In the eukaryotic cell, DNA replication occurs at multiple origin sites that are recognised and bound by a six-subunit assembly called the origin recognition complex (ORC) (Bell and Stillman 1992). At the onset of mitosis, the coordinated activation of the origins (known as licensing) culminates with the loading of the mini-chromosome maintenance (MCM) hetero-hexameric complex, which is believed to function as the replicative DNA helicase (Bell and Dutta 2002). In contrast, replication initiation in the bacterial cell is typically controlled by a single protein (DnaA in *Escherichia coli*), which binds repeated sequence elements (known as DnaA-boxes) within a single chromosomal origin, *oriC* (Fuller et al. 1984).

Living organisms have evolved different strategies to recruit and load DNA helicases at replication origins, and accessory factors, referred to as helicase-loaders, are required to accomplish this task (Davey and O'Donnell 2003; Konieczny 2003). The helicase-loading process was best characterized at a molecular level in two

bacterial systems: *E. coli* (Davey et al. 2002) and *Bacillus subtilis* (Velten et al. 2003). The *E. coli* replicative homo-hexameric DNA helicase DnaB, associates with its loader DnaC and forms a 6:6 complex that is recruited at the *oriC* by the initiator protein DnaA (Kornberg and Baker 1992b). DnaB is then released in an active form and this initiates the formation of two replication forks that extend in opposite directions from the *oriC* (Fang et al. 1999). Since *E. coli* DnaB forms stable hexamers in solution, which are unable to efficiently self-load onto a single stranded DNA, it was proposed that its loading by DnaC be carried out by a ring-breaking mechanism (Davey and O'Donnell 2003). In *B. subtilis*, the replicative DNA helicase DnaC forms homo-hexamers in solution in the presence of ATP, but in the absence of ATP the hexameric form readily dissociates into monomers. The pre-formed hexameric DnaC helicase is unable to unwind DNA, and must be assembled from monomers around DNA by a loading system that consists of two proteins, DnaI and DnaB (Velten et al. 2003). In the eukaryotic cell, the replicative DNA helicase-loading process requires the cell division control (Cdc6) proteins 6 and 45 (Cdc45), as well as the factor Ctd1 (Bell and Dutta 2002). These initiation factors promote the association of the MCM complex to the ORC-bound replication origins by a molecular mechanism that has not yet been completely elucidated.

In most archaea there is a single MCM protein, which forms homo-hexamers (or double-hexamers) in solution (De Felice et al. 2004b; Fang et al. 1999; Fuller et al. 1984; Gabrowski and Kelman 2001; Grabowski and Kelman 2003). Some archaeal species, such as those belonging to the *Pyrococcus* genus, were found to possess a single putative homolog of the eukaryotic initiation factors Cdc6 and ORC subunit 1 (ORC1). In contrast, other archaeal species have multiple putative Cdc6/ORC1-like factors (two homologs are present in *Methanobacterium thermoautotrophicum*, three homologs in *Sulfolobus solfataricus*, and even ten putative homologs in *H. volcanii*; (see Grabowski and Kelman 2003 for a review). In the species where a single Cdc6/ORC1-like factor is present, it is likely that it may function both as a replication initiator and an MCM-loader. On the other hand, in the archaeal species, where multiple Cdc6/ORC1-like proteins have been identified, origin-recognition/binding and MCM-loading functions may be carried out by distinct Cdc6/ORC1 homologs. Nonetheless, in several archaeal species, the number of Cdc6/ORC1-like proteins seems to correlate with the number of replication origins. In fact, DNA replication is initiated from a single origin in *Pyrococcus* sp. (Myllykallio et al. 2000), whereas three active DNA replication origins are present in *Sulfolobus* (Robinson et al. 2004; Lundgren et al. 2004) and multiple origins are found in *H. volcanii* (Berquist and DasSarma 2003). It has been clearly demonstrated that in certain archaeal species, the Cdc6/ORC1-like proteins are able to specifically bind the replication origins either in vivo or in vitro, suggesting that they could truly act as replication initiators (Robinson et al. 2004; Matsunaga et al. 2001). In contrast, direct evidence that the archaeal Cdc6/ORC1-like factors promote recruitment of the MCM-like DNA helicase at the replication origins has not yet been reported.

In previous studies, the biochemical properties of Sso Cdc6-2, one of the three Cdc6/ORC1-like factors from the hyperthermophilic crenarchaeon *S. solfataricus* (P2 strain, De Felice et al. 2003, 2004a, 2004b), were investigated. It was reported that Sso Cdc6-2 is a monomer in solution, binds single-stranded and double-stranded DNA molecules, undergoes auto-phosphorylation in vitro, strongly inhibits the unwinding activity of the homo-hexameric MCM-like DNA helicase from *S. solfataricus* (Sso MCM), stimulates the Sso MCM DNA-binding function and physically interacts with it (De Felice et al. 2003, 2004b). In addition, we demonstrated that Sso Cdc6-2 has a modular organization, since a C-terminally deleted form of the protein (named ΔC) retains the ability to bind and hydrolyse ATP, whereas the C-terminal Winged Helix (WH)-domain, produced as a separate recombinant protein (named ΔN), is able to bind single-stranded and double-stranded DNA and to inhibit the Sso MCM DNA helicase activity (De Felice et al. 2004a).

This study has been focused on the biochemical characterization of the two additional *S. solfataricus* Cdc6/ORC1-like factors that are referred to as Sso Cdc6-1 and Cdc6-3.

Materials and methods

Materials

All chemicals were of reagent grade. The restriction and modification enzymes were from New England Biolabs. Radioactive nucleotides were purchased from Amersham Biosciences (Buckinghamshire, UK) And the oligonucleotides synthesized by Proligo (Paris, France).

Proteins

The Sso MCM (Carpentieri et al. 2002) and wild type and KA mutant Sso Cdc6-2 were purified as previously described (De Felice et al. 2003).

Cloning of Sso Cdc6-1 and Cdc6-3

The genes, coding for Sso Cdc6-1 and Cdc6-3, were amplified from the *S. solfataricus* P2 genomic DNA using the High Fidelity PCR system (Roche Applied Science, USA). The Sso Cdc6-1 gene was amplified with the following oligonucleotides: Cdc6-1-for (5′-GGTTGAATTCATGAGTGATATAATTGATGAGGTCATTTCT-3′) as the 5′ primer (the engineered *Eco*RI site is underlined) and oligonucleotide Cdc6-1-rev

(5′-GGTTCTGCAGTCAACTCCAGAGATCAGCA-AACCTACTATC-3′) as the 3′ primer (the engineered *Pst*I site is underlined). The PCR product was cloned into the *Eco*RI/*Pst*I-linearized *E. coli* expression vector pProEX-Hta (*GIBCO*-BRL), to create the construct named pProEX-Hta-SsoCdc6-1, and sequenced.

The oligonucleotides used to amplify the Sso Cdc6-3 gene were as follows: Cdc6-3-for (5′-TTGGGA-ATTCGTGCATGTGATAAGGGAAACATTGAAA-GGT-3′) as the 5′ primer (the engineered *Eco*RI site is underlined) and Cdc6-3-rev (5′-TTGGCTGCAGTT-AATTTTCTCCCACATCATCAAAATCACC-3′) as the 3′ primer (the engineered *Pst*I site is underlined). The PCR product was cloned into the *Eco*RI/*Pst*I-linearised *E. coli* expression vector pProEx-Hta (*GIBCO*-BRL), to create the construct named pProEX-Hta-SsoCdc6-3, and sequenced. Since the Sso Cdc6-3 protein was poorly expressed, we decided to clone the corresponding gene into pET-28a (Novagen). The Sso Cdc6-3 gene was recovered, by digesting the pProEX-Hta-SsoCdc6-3 with *Nco*I and *Xho*I, and inserted into the *Nco*I/*Xho*I-linearised pET-28a vector to create the construct named pET-28a-SsoCdc6-3.

Expression and purification of the recombinant proteins

E. coli BL21-CodonPlus(DE3)-RIL cells (Stratagene, CA, USA), transformed with the plasmid pProEX-SsoCdc6-1, were grown at 37°C in 1 l of LB medium containing 30 μg/ml chloramphenicol and 100 μg/ml ampicillin. When the culture reached a D_{600} of 0.7, protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.2 mM. The bacterial culture was incubated at 37°C for an additional 2 h. The cells were then harvested by centrifugation, and the pellet stored at -20°C until use.

The pellet was thawed and re-suspended in 40 ml of buffer A (25 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 100 mM NaCl, 30% glycerol, and 2 mM imidazole) and supplemented with some protease inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 0.2 μg/ml benzamidine, 1 μg/ml aprotinin). the cells were broken by two consecutive passages through a French pressure cell apparatus (Aminco Co., Silver Spring, MD, USA) at 1500 psi (1 psi = 6.9 kPa). The resulting cell extract was centrifuged for 30 min at 30,000 rpm (Beckman 60 Ti rotor) at 10°C. The supernatant was subjected to heat-treatment at 70°C for 5 min, and then incubated in ice for 10 min. The thermo-precipitated proteins were removed by centrifugation for 30 min at 30,000 rpm (Beckman 60 Ti rotor) at 10°C. The supernatant was passed through a 0.22 μm filter (Millipore, MA, USA) and loaded onto a Ni-NTA (Ni^{2+}-nitrilotriacetate) superflow-agarose column (Qiagen, CA, USA), pre-equilibrated in buffer A. After a washing step with buffer A, the elution was carried out with 60 ml of an imidazole stepwise gradient (50–500 mM) in buffer A. 1.5 ml fractions were collected and analysed by SDS-PAGE to detect the Sso Cdc6-1 polypeptide. Fractions containing the recombinant protein were pooled and centrifuged for 10 min at 30,000 rpm (Beckman 60 Ti rotor) to remove precipitated material that appeared shortly after elution from the column. The supernatant was dialysed overnight against buffer A. The dialysed sample (volume: 10 ml) was divided into small aliquots (volume: 50 μl), which were stored at −20°C. The final yield of the recombinant protein after this purification procedure was about 10 mg.

E. coli BL21-CodonPlus(DE3)-RIL cells (Stratagene), transformed with the plasmid pET-28a-SsoCdc6-3, were grown at 37°C in 1 l of LB medium containing 30 μg/ml chloramphenicol and 30 μg/ml kanamycin. When the culture reached a D_{600} of 0.7 optical density, protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.2 mM. The bacterial culture was incubated at 37°C for an additional 2 h. The cells were then harvested by centrifugation and the pellet stored at −20°C until use.

The pellet was thawed and re-suspended in 40 ml of buffer B (25 mM Tris–HCl, pH 6.8, 2.5 mM $MgCl_2$, 50 mM NaCl, 15% glycerol), supplemented with a Protease Inhibitors Cocktail (Sigma, MO, USA). the cells were broken by two consecutive passages through a French pressure cell apparatus (Aminco Co., Silver Spring, MD, USA) at 1500 psi (1 psi = 6.9 kPa). The resulting crude extract was centrifuged for 30 min at 30,000 rpm (Beckman 60 Ti rotor) at 10°C. The supernatant was subjected to heat-treatment at 70°C for 10 min, and then incubated in ice for 10 min. The thermo-precipitated proteins were removed by centrifugation for 30 min at 30,000 rpm (Beckman 60 Ti rotor) at 10°C. The supernatant was passed through a 0.22 μm filter (Millipore) and loaded onto a MonoS column (HR 10/10, Amersham Biosciences) equilibrated in buffer B. A 70 ml linear gradient of NaCl in buffer B (0.05–1.0 M, flow rate: 0.5 ml/min) was applied to the column. Sso Cdc6-3 eluted at a NaCl concentration of about 0.1 M. The fractions containing the protein were pooled (volume: 6 ml). This sample was divided into small aliquots (volume: 100 μl), which were stored at −20°C. The final yield of the recombinant protein after this purification procedure was about 40 mg.

Gel filtration chromatography

Samples of the purified Sso Cdc6-1 and Sso Cdc6-3 (50 μg in 100 μl) were subjected to analytical gel filtration chromatography on a Superose 6 HR 10/30 column (Amersham Biosciences), equilibrated with buffer C (25 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$). The chromatographic run was carried out at a flow rate of 0.3 ml/min at room temperature. The column was calibrated by running a set of gel filtration markers that included: tyroglobulin (669 kDa), ferritin (440 kDa), BSA (69 kDa), and ribonuclease A (13.7 kDa).

DNA substrates

Oligonucleotides were labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP. To prepare double stranded substrates, the labelled oligonucleotide was annealed to a threefold molar excess of a cold complementary strand. Substrates containing a bubble made from duplex surrounding poly dT (Bub-20T), had the following sequences: 5′-TCTACCTGGACGACCGGG$(T)_{20}$G-GGCCAGCAGGTCCATCA-3′. Forked (Fork) and tailed duplex substrates were constructed by annealing the appropriate combinations of the following oligonucleotides: 5′-GCTCGGTACCCGGGGATCCTCT-AGA(T)n-3′, 5′-$(T)_n$TCT-AGAGGATCCCCGGGTA-CCGAGC-3′, where $n=0$ or 20. When $n=0$ for both substrates, a duplex of 25 bp was produced. The oligonucleotide (5′-CCCAGTCACGACGTTGTAAAAC-GACGGCCAGTGCGAGGCGCGCGAAGACCG-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 which contained: 200 fmol of [^{32}P]-labelled DNA in 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5 mM $MgCl_2$, and 0.7 mM 2-mercaptoethanol, and the indicated amounts of Sso Cdc6-1 or Cdc6-3 and/or Sso MCM. Following incubation for 10–15 min at room temperature, the complexes were separated by electrophoresis through 5% polyacrylamide/bis gels (37.5:1) in 0.5 time TBE. The gels were dried down and analysed with a Storm PhosphorImager, using the ImageQuant software (Molecular Dynamics).

In vitro auto-phosphorylation of the Sso Cdc6 proteins

Samples of purified Sso Cdc6-1, Cdc6-2, and Cdc6-3 or Sso Cdc6-2 KA (1 µg), were incubated for 30 min at 70°C in a reaction mixture (volume: 20 µl) containing 1.66 pmol of [γ-^{32}P]ATP in 25 mM Tris–HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, 5 mM $MgCl_2$. The proteins were then separated on SDS-10% PAGE, and ^{32}P-labeled bands were analysed using a Storm PhosphorImager (Molecular Dynamics).

DNA helicase activity assays

A 85-mer oligonucleotide was used for the preparation of the DNA helicase substrate as previously described (Carpentieri et al. 2002). This oligonucleotide (5′-TTGAACCACCCCCTTGTTAAATCACTTCTACTT-GCATGCCTGCAGGTCGACTCTAGAGGATCCC-CGGGTACCGAGCTCGAATTCG) was complementary to the M13mp18(+) strand, except for a 30-nt 5′-tail (the tail is underlined). Helicase assay reaction mixtures (20 µl) contained 50 fmol of ^{32}P-labelled substrate (about 1×10^3 cpm/fmol) in 25 mM Tris–HCl, pH 8.0, 2.5 mM 2-mercaptoethanol, 50 mM sodium acetate, and 5 mM $MgCl_2$. The reactions were incubated for 30 min at 70°C, in a heated-top PCR machine, to prevent evaporation and stopped by the addition of 5 µl of five times stop solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml proteinase K, 20% glycerol, 0.1% bromophenol blue), then run on a 8% polyacrylamide gel in TBE containing 0.1% SDS at a constant voltage of 150 V. After electrophoresis, the gel was soaked in 20% trichloroacetic acid and analysed with a Storm PhosphorImager (Molecular Dynamics).

Surface plasmon resonance measurements

Interaction among the Sso Cdc6 factors was monitored using the Biacore 2000 system (Biacore, Sweden). Sso Cdc6-1, Cdc6-2, and Cdc6-3 was coupled to the carboxymethylated dextran-modified gold surface of a CM5 sensor chip, as described in the Biacore system's manual. Samples of each Sso Cdc6 factor were diluted in buffer HBS, at the indicated protein concentrations, and passed over the sensor surface at a flow rate of 10 µl/min.

Results

Purification of Sso Cdc6-1 and Cdc6-3

Sso Cdc6-1 was overproduced in *E. coli* as a soluble hexa-histidine-tagged protein, using the plasmid pProEX-Hta, and purified using a procedure that included thermal treatment of the cell extract and a chromatographic step on a Ni^+-chelate column, as described under Materials and methods. In contrast, we found that Sso Cdc6-3 was poorly expressed as a hexa-histidine-tagged protein in *E. coli*, using the above expression vector. Therefore, we decided to clone the Sso Cdc6-3 gene into the pET-28a plasmid (Novagen), without any additional tag. The recombinant protein was expressed at a high level in the soluble form. A purification protocol was set up that included thermal treatment of the *E. coli* cell extract and a chromatographic step on a MonoS column, as described under Materials and methods.

A Coomassie-stained SDS-polyacrylamide gel of the purified Sso Cdc6 factors is shown in Fig. 1a. The lower mobility of the recombinant Sso Cdc6-1 is due to the presence of the hexa-histidine tag at the N-terminus end of the protein. The identity of the two recombinant polypeptides was assessed by N-terminal sequence analyses (data not shown). A Stokes radius of about 35 and 39 Å was estimated for Sso Cdc6-1 and Cdc6-3, respectively, by gel filtration analyses of the purified proteins (see Fig. 1b). These findings suggest that Sso Cdc6-1 and Cdc6-3 are monomers in solution.

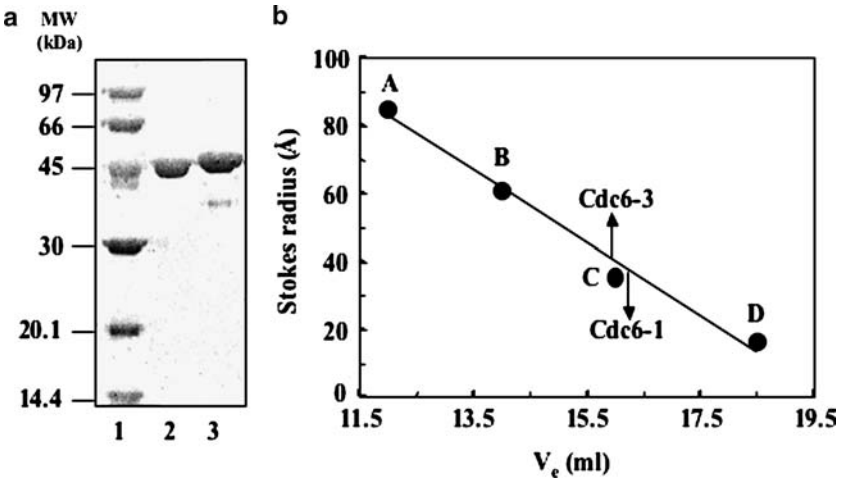

Fig. 1 Purification and gel filtration analysis of Sso Cdc6-1 and Cdc6-3. **a** Coomassie blue-stained SDS-polyacrylamide gel of purified Sso Cdc6-1 and Cdc6-3 (10 μg; *lanes 3 and 2*, respectively). **b** Samples of Sso Cdc6-1 and Cdc6-3 (50 μg of each purified protein in 100 μl) were subjected to gel filtration on a Superose 6 column, as described under Materials and methods. The column was calibrated with the following standard proteins: *A* tyroglobulin (Stokes radius: 85.0 Å, V_e: 12 ml); *B* ferritin (Stokes radius: 61.0 Å, V_e: 14 ml); *C* bovine serum albumin (Stokes radius: 35.5 Å, V_e: 16 ml); *D* ribonuclease A (Stokes radius: 16.4 Å, V_e: 18.5 ml). A plot is reported of the Stokes radius versus the elution volume of each standard protein

Auto-phosphorylation of Sso Cdc6-1 and Cdc6-3

As it has been previously reported, Sso Cdc6-2 is able to auto-phosphorylate in vitro (De Felice et al. 2002), similarly to the *Methanobacterium thermoautotrophicum* (Mth) Cdc6 proteins (Gabrowski and Kelman 2001). It was found that even Sso Cdc6-1 was able to auto-phosphorylate when incubated with [γ-^{32}P]ATP at 70°C (Fig. 2, *lane 3*), whereas Sso Cdc6-3 did not show this capability (Fig. 3, *lane 2*). In this set of experiments samples of the wild type and KA mutant Sso Cdc6-2 were used in positive and negative auto-phosphorylation control reactions (Fig. 2, *lanes 1* and *4*), respectively. The auto-phosphorylation activity of the Sso Cdc6 proteins, in the presence of DNA, was also tested; but no significant effect of either single-stranded or double-stranded DNA molecules was observed (unpublished results), similarly to what had been previously reported for the Sso Cdc6-2 protein (De Felice et al. 2003).

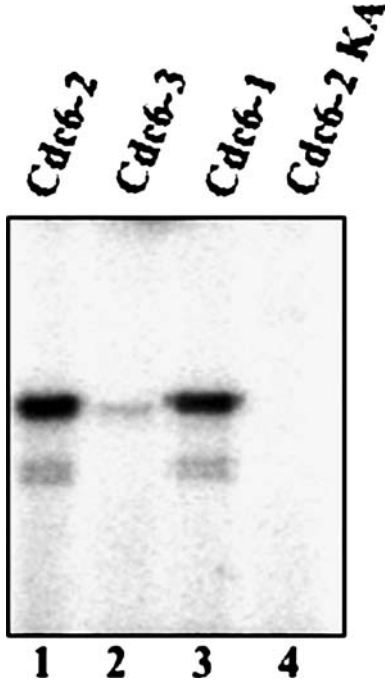

Fig. 2 Auto-phosphorylation of the Sso Cdc6 proteins. Each indicated protein (1 μg) was incubated at 70°C for 30 min in a mixture (20 μl) containing [γ-^{32}P]ATP, as described under Materials and methods. After incubation, the samples were subjected to SDS-PAGE. The radioactive bands were visualized using a Storm Phosphorimager (Molecular Dynamics)

DNA binding activity of Sso Cdc6-1 and Cdc6-3

The ability of Sso Cdc6-1 or Cdc6-3 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), 5′-tailed or 3′-tailed duplexes (5′-tail and 3′-tail, data not shown), blunt double-stranded molecules (no-tail), and single-stranded oligonucleotides (ssDNA). As shown in Fig. 3, both Sso Cdc6-1 and Sso Cdc6-3 bind DNA molecules that contain a bubble, or a fork, or a tail, and produce various shifted bands, whose intensity is dependent upon the protein concentration. Sso Cdc6-3 binds the Bubble-20T and the Fork molecules with a higher affinity, when compared to Sso Cdc6-1. On the other hand, the 5′-tail and 3′-tail ligands, as well as the single-stranded or blunt double-stranded oligonucleotides, were bound with similar low affinity by both the Sso Cdc6-1 or Cdc6-3

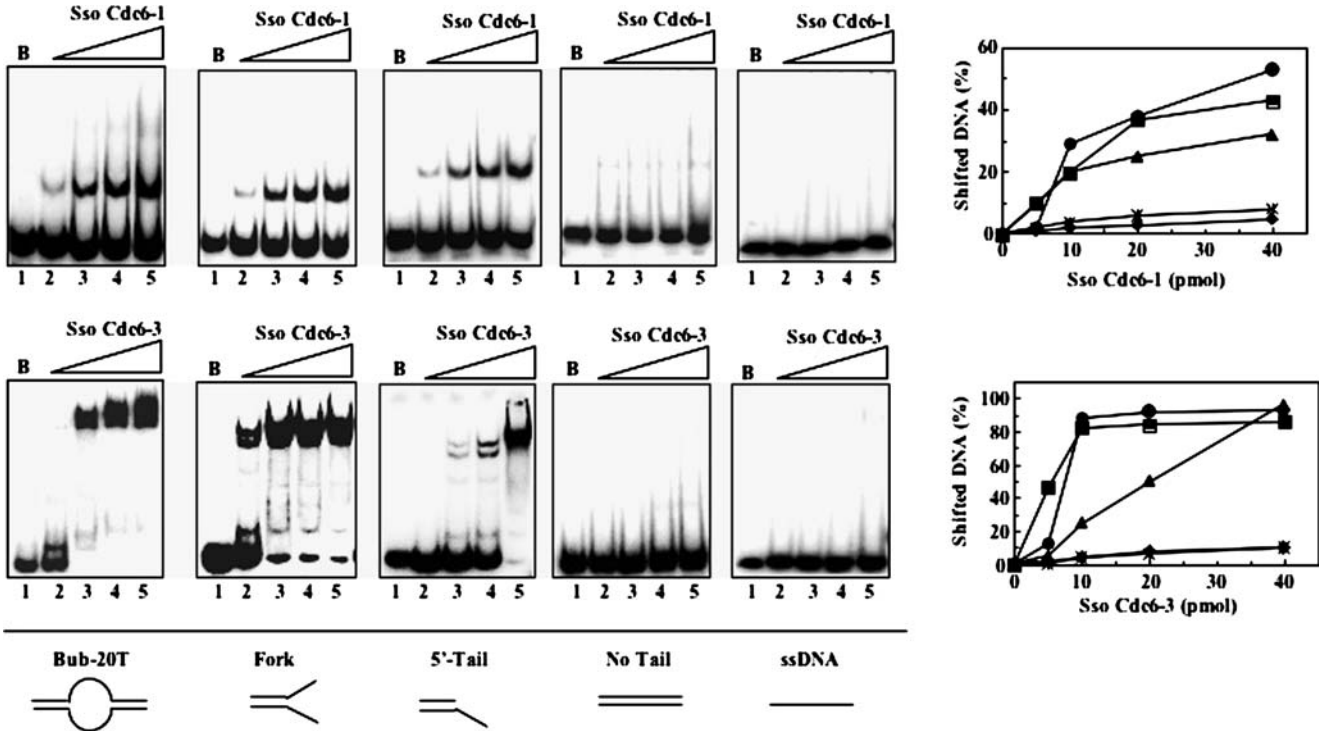

Fig. 3 DNA-binding activity of Sso Cdc6-1 and Cdc6-3 on various DNA molecules. DNA band shift assays were carried out on the DNA molecules, schematically depicted in the *bottom part*, using increasing amounts of the indicated Sso Cdc6 protein (from 5 to 40 pmol in a volume of 10 µl, *lanes 2–5* in each gel), as described under Materials and methods. The lanes marked with *B* were loaded with control samples without protein. A plot of the shifted DNA versus the amount of each Sso Cdc6 protein used is shown. Symbols used are: *circles* (Bubble-20T), *squares* (Fork), *triangles* (5′-tail), *diamonds* (no-tail)

(see Fig. 3 and data not shown). These findings indicate that both Sso Cdc6-1 and Cdc6-3 are able to bind DNA in a structure-dependent manner, preferring DNA molecules that resemble early replication intermediates. Similar behaviour was observed in a previous report on the biochemical characterization of the Sso Cdc6-2 factor and the Sso MCM homo-hexameric complex (De Felice et al. 2004b).

Sso Cdc6-1 and Cdc6-3 stimulate binding of Sso MCM to bubble-containing or fork-containing DNA molecules

It has been previously found that Sso Cdc6-2 stimulates the binding of the Sso MCM DNA helicase to bubble-containing DNA molecules (De Felice et al. 2004b). This prompted an analysis of the effect of Sso Cdc6-1 and Cdc6-3 on the DNA-binding activity of Sso MCM. When amounts of Sso Cdc6-1 or Cdc6-3, ranging from 0 to 8 pmol (sample volume: 10 µl), were incubated with the Bubble-20T ligand, a low level of DNA band-shift was observed in electrophoretic mobility shift assays (Fig. 4, *lanes 2–4* of each gel). As previously reported, Sso MCM binds DNA with structural specificity and shows a high affinity for the T-bubble ligand (De Felice et al. 2004b). At low protein concentrations (ranging from 0.01 to 0.1 pmol in a volume of 10 µl), binding of the Sso MCM to this DNA ligand produced various retarded bands; whereas, at higher protein concentrations, the faster migrating bands disappeared and only a lower mobility complex was observed (De Felice et al. 2004b). Since Sso MCM hexamers are not stable and dissociate upon dilution (Carpentieri et al. 2002), it is likely that the faster migrating bands were produced by binding to the DNA of these various Sso MCM sub-assemblies; whereas, the low-mobility complexes, that are formed at the higher protein concentrations, could contain the protein hexameric form. This study performed electrophoresis mobility shift assays in the presence of a Sso MCM concentration (0.1 pmol in a volume of 10 µl, see Fig. 4, *lane 5* of each gel) that produced the faster migrating protein–DNA complexes and only a minimal amount of the above lower mobility species (indicated as Complex I in Fig. 4). When either Sso Cdc6-1 or Cdc6-3 and Sso MCM were mixed together in the above experimental conditions, we observed a gradual increase of Complex I with respect to experiments in which Sso MCM was incubated alone, and this increase was found to be proportional to the concentration of the Sso Cdc6-1 or Cdc6-3 used (Fig. 4, *lanes 6–8* of each gel). The formation of Complex I was increased by about 40-folds, when 8 pmol of each Sso Cdc6 factor was used (see plot in Fig. 4). These results indicate that either Sso Cdc6-1 or Sso Cdc6-3 promote Sso MCM binding to bubble-containing oligonucleo-

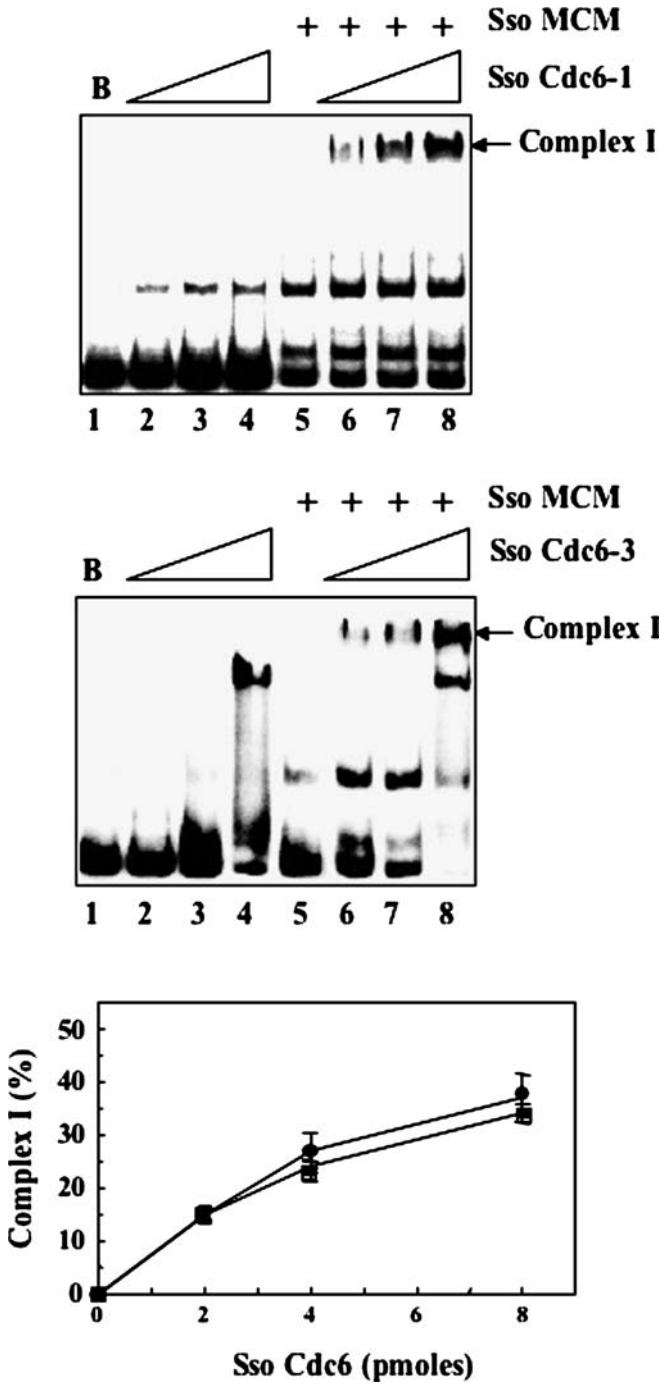

Fig. 4 Effect of Sso Cdc6-1 and Cdc6-3 on the Sso MCM DNA-binding activity. DNA band shift assays were carried out on the bubble-containing DNA molecules using increasing amounts of the indicated Sso Cdc6 protein (from 2 to 8 pmol) in the absence (*lanes 2–4*) or in the presence (*lanes 6–8*) of Sso MCM at 0.1 pmol in a volume of 10 µl, as described under materials and methods. The lanes marked with *B* were loaded with control samples without protein. A plot is reported of the percentage of probe shifted as the Complex I in each lane versus the amount of the Sso Cdc6 protein used. Symbols used are: *squares* and *circles* (Sso Cdc6-1 and Cdc6-3, respectively)

tides, as previously reported for the Sso Cdc6-2 factor (De Felice et al. 2004b). 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 (data not shown). Furthermore, this effect was not dependent on the order of the addition of the two proteins to the mixtures containing the DNA molecules, as also reported for Sso Cdc6-2 (De Felice et al. 2004b).

Effect of Sso Cdc6-1 and Cdc6-3 on the Sso MCM DNA helicase activity

A previous report had found that the Sso MCM DNA helicase activity significantly inhibited by the Sso Cdc6-2 (De Felice et al. 2003). Therefore, it was decided to test whether the Sso Cdc6-1 and the Cdc6-3 had any effect on the unwinding activity of the Sso MCM DNA. Strand-displacement assays were performed at 70°C for 30 min. The substrate utilised was prepared by annealing to M13mp18 ssDNA, a ^{32}P-labelled synthetic oligonucleotide of 85 nucleotides, which gave rise to partial duplexes having a 30 nt 5′-tail. The helicase activity of Sso MCM was assayed in the presence of increasing amounts of Sso Cdc6-1 or Cdc6-3 (see Fig. 5, *lanes 3–7* of each gel). When Sso Cdc6-1 was added at a concentration of 5 pmol/µl, the Sso MCM unwinding activity was almost completely inhibited. Sso Cdc6-3 also exerted an inhibitory effect on the Sso MCM DNA helicase activity, although less striking with respect to that observed in the presence of Sso Cdc6-1. Similar results were also obtained when a substrate with a 9 nt 5′-tail was used in the DNA helicase assays (data not shown).

Physical interaction of the Sso Cdc6 factors

Physical interaction among the three *S. solfataricus* Cdc6 factors was tested using the BIAcore system. It was found that Sso Cdc6-3 was able to interact with sensorchips immobilised Sso Cdc6-1 and Cdc6-2, as described under Materials and methods. Figure 6 shows typical overlaid sensorgrams, detected with increasing concentrations of Sso Cdc6-3 (0.5–4.0 µM) as the analyte. However, these associations were found to be unstable and were not detected by other techniques (such as gel filtration analyses of protein mixtures, co-immuno-precipitation experiments and pull-down assays on various mixtures of his-tagged and non-his-tagged recombinant proteins; data not shown). No specific interaction between Sso Cdc6-1 and Cdc6-2, either by surface plasmon resonance measurements or pull-down experiments, was observed.

Discussion

The hyperthermophilic crenarchaeon *S. solfataricus* (P2 strain) possesses three Cdc6/ORC1-like factors, which were named Sso Cdc6-1, Cdc6-2 and Cdc6-3 (She et al.

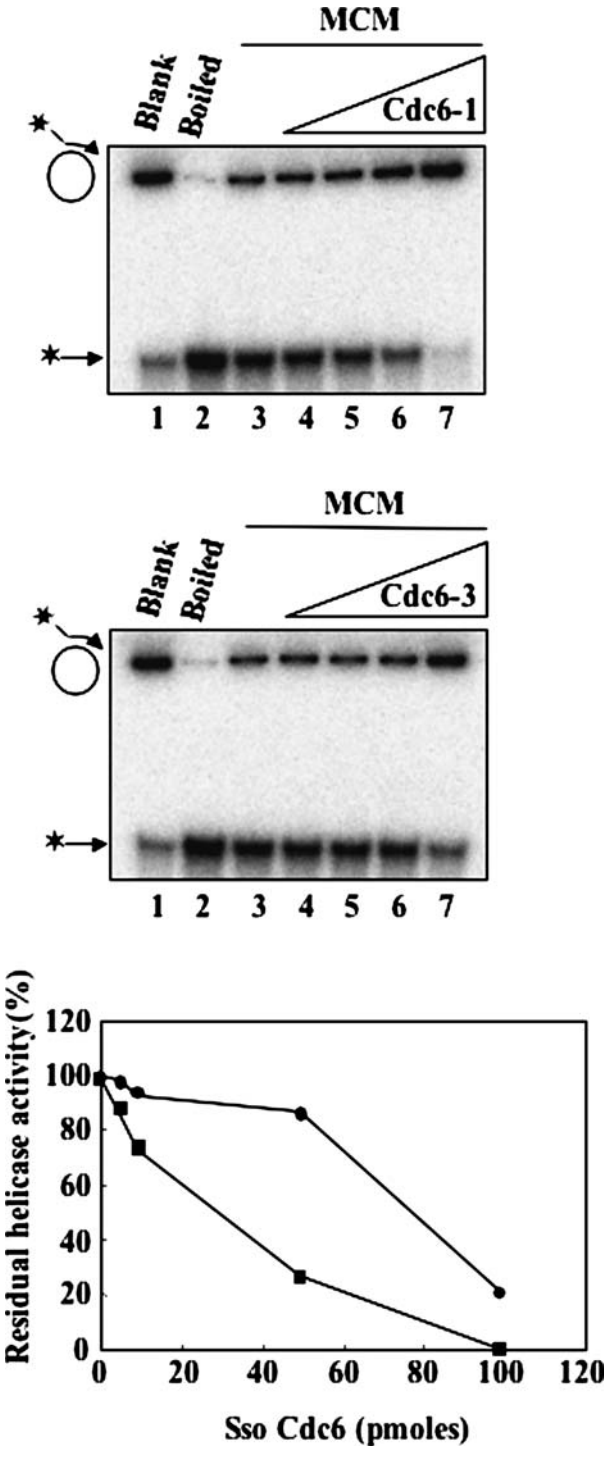

Fig. 5 Effect of Sso Cdc6-1 and Cdc6-3 on the Sso MCM DNA helicase activity. The DNA unwinding activity of Sso MCM (700 ng, 1.5 pmol of hexamer) was tested at 70°C for 30 min in the presence of 0, 5, 10, 50, and 100 pmol of the indicated Sso Cdc6 protein (*lanes 3–7)* in a volume of 20 µl. A plot is reported of the residual Sso MCM DNA helicase activity versus the amount of the Sso Cdc6 protein present in each enzymatic assay. Symbols used are: *squares* and *circles* (Sso Cdc6-1 and Cdc6-3, respectively). The unwinding activity of Sso MCM alone was taken as 100%. Results of a typical experiment are shown

2001). Previous studies have demonstrated that the Sso Cdc6-2 factor inhibits the DNA helicase activity of Sso MCM (De Felice et al. 2003), but stimulates Sso MCM binding to bubble-containing and fork-containing DNA oligonucleotides that mimic replication intermediates (De Felice et al. 2004b). This work is a report on the biochemical characterization of Sso Cdc6-1 and Cdc6-3. These proteins were produced in soluble form in *E. coli* and purified to homogeneity. It has been found that both Sso Cdc6-1 and Cdc6-3 behave as monomers in solutions by gel filtration chromatography. The ability of these proteins to bind DNA in a structure-dependent fashion was analysed and it was found that both factors show a clear preference for DNA molecules bearing a bubble or a fork or a tail, as previously reported for Sso Cdc6-2 (De Felice et al. 2004b). In addition, both Sso Cdc6-1 and Cdc6-3 inhibit the DNA unwinding activity of Sso MCM, although they strongly stimulate the interaction of Sso MCM with bubble-containing synthetic oligonucleotides. Moreover, like the Sso Cdc6-2 (De Felice et al. 2003), Sso Cdc6-1 is also able to auto-phosphorylate in vitro, whereas Sso Cdc6-3 does not possess this ability. The inability to auto-phosphorylate in vitro is a peculiar feature of Sso Cdc6-3, with respect to both Sso Cdc6-1 and Cdc6-2. It is not clear whether this different behaviour of Sso Cdc6-3 has any biological significance. It has been reported that the Cdc6/ORC1 homologs from *M. thermoautotrophicum* (Gabrowski and Kelman 2001), *Pyrobaculum aerophilum* (Gabrowski and Kelman 2001) and *Aeropyrum pernix* (Singleton et al. 2004) are able to auto-phosphorylate in vitro. However, the crystal structure of *P. aerophilum* (Liu et al. 2000) and *A. pernix* (Singleton et al. 2004) Cdc6/ORC1-like factors did not show any evidence of phosphorylated residues.

The three *Sulfolobus* Cdc6/ORC1-like factors were shown to possess the ability to bind specific sequence boxes, within the *S. solfataricus* replication origins in vivo and in vitro (Robinson et al. 2004). It was reported that the chromosome of this species possesses three active replication origins (Robinson et al. 2004; Lundgren et al. 2004). Two of them were finely mapped and found to be located in front of the genes coding for Sso Cdc6-1 (*oriC1*) and Sso Cdc6-3 (*oriC2*). Sso Cdc6-1 and Cdc6-2 were reported to bind to both origins, whereas Sso Cdc6-3 binds only *oriC2* (Robinson et al. 2004). It has been found, by surface plasmon resonance measurements, that Sso Cdc6-3 is able to establish a physical interaction with Cdc6-1 and Cdc6-2. However, we were unable to detect these associations by other techniques (such as gel filtration analyses and co-immuno-precipitation experiments). Using Western blot analysis with anti-sera raised against the *S. solfataricus* Cdc6/ORC1-like factors, Robinson et al. (2004) showed that, in synchronised cultures of the related species *Sulfolobus acidocaldarius*, the Cdc6-2 homolog is present only in stationary phase cells, whereas the other two Cdc6/ORC1-like factors

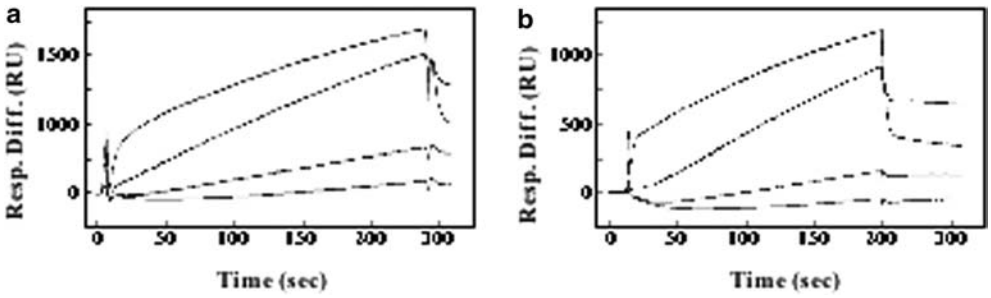

Fig. 6 Analysis of Sso Cdc6-3 interaction with Sso Cdc6-1 and Cdc6-2 by surface plasmon resonance experiments. Overlaid plots of sensorgrams of Sso Cdc6-3 increasing concentrations (*lower* to *upper curve* in **a** and **b**: 0.5, 1.0, 2.0 and 4.0 μM) injected at 10 μl/min over the Sso Cdc6-1 (**a**) and the Sso Cdc6-2 (**b**) surface of the sensor chip

were mainly expressed in the logarithmic phase. Based on these findings, the authors have proposed that Sso Cdc6-1 and Cdc6-3 promote DNA replication initiation, whereas Sso Cdc6-2 acts as a negative regulator of this process by not dividing cells.

On the basis of primary structure alignments, the archaeal Cdc6/ORC1-like factors have been classified into two major subfamilies (Berquist and DasSarma 2003; Singleton et al. 2004). An important difference between these two classes of proteins seems to reside in the sequence of the WH-domain, which is responsible for the DNA-binding function in various archaeal Cdc6/ORC1-like factors (Singleton et al. 2004). In particular, the Cdc6/ORC1 homologs of the subfamily I (including Sso Cdc6-1 and possibly Sso Cdc6-3) contain a conserved motif, in the turn and recognition helix of the WH domain, that has been suggested to directly interact with the major groove of the DNA target sequence (Capaldi and Berger 2004). In contrast, this sequence motif is changes among the members of the subfamily II (including Sso Cdc6-2), raising the possibility that these factors bind DNA by a different mechanism that involves the ''wing'' portion of the WH domain (Singleton et al. 2004).

It is clear that the establishment of an in vitro DNA replication system is required to determine the precise role of each Cdc6/ORC1-like factor in the initiation process and whether other, still unknown, factors are required. Our data indicates that the three *Sulfolobus* Cdc6/ORC1-like factors bind DNA with structural specificity, preferring bubble-containing and fork-containing oligonucleotides, and also stimulate the binding of Sso MCM to these DNA molecules in a sequence-independent fashion. This finding raises the intriguing possibility that these proteins may also play a role in resuming stalled DNA replication forks during chromosome duplication.

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