Characterization of the Replication Initiator Orc1/Cdc6 from the Archaeon *Picrophilus torridus*

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Eukaryotic DNA replication is preceded by the assembly of prereplication complexes (pre-RCs) at or very near origins in G₁ phase, which licenses origin firing in S phase. The archaeal DNA replication machinery broadly resembles the eukaryal apparatus, though simpler in form. The eukaryotic replication initiator origin recognition complex (ORC), which serially recruits Cdc6 and other pre-RC proteins, comprises six components, Orc1-6. In archaea, a single gene encodes a protein similar to both the eukaryotic Cdc6 and the Orc1 subunit of the eukaryotic ORC, with most archaea possessing one to three Orc1/Cdc6 orthologs. Genome sequence analysis of the extreme acidophile *Picrophilus torridus* revealed a single Orc1/Cdc6 (PtOrc1/Cdc6). Biochemical analyses show MBP-tagged PtOrc1/Cdc6 to preferentially bind ORB (origin recognition box) sequences. The protein hydrolyzes ATP in a DNA-independent manner, though DNA inhibits MBP-PtOrc1/Cdc6-mediated ATP hydrolysis. PtOrc1/Cdc6 exists in stable complex with PCNA in *Picrophilus* extracts, and MBP-PtOrc1/Cdc6 interacts directly with PCNA through a PIP box near its C terminus. Furthermore, PCNA stimulates MBP-PtOrc1/Cdc6-mediated ATP hydrolysis in a DNA-dependent manner. This is the first study reporting a direct interaction between Orc1/Cdc6 and PCNA in archaea. The bacterial initiator DnaA is converted from an active to an inactive form by ATP hydrolysis, a process greatly facilitated by the bacterial ortholog of PCNA, the β subunit of Pol III. The stimulation of PtOrc1/Cdc6-mediated ATP hydrolysis by PCNA and the conservation of PCNA-interacting protein motifs in several archaeal PCNAs suggest the possibility of a similar mechanism of regulation existing in archaea. This mechanism may involve other yet to be identified archaeal proteins.

The complex process of DNA replication has many tiers of regulation. In bacteria, the process initiates from a single origin, while in eukaryotes, initiation occurs from multiple origins. DNA synthesis is preceded by origin recognition by one or more initiator proteins. The bacterial initiator DnaA interacts with the single origin *oriC*, and in eukaryotes, the ORC (origin recognition complex), comprising Orc1-6, binds to origin DNA and subsequently recruits other pre-RC (prereplication complex) components such as Cdc6, Cdt1, MCM2-7, and others in an orderly manner. The assembly of pre-RCs licenses origins to fire as cells enter S phase. Archaea, the third domain of life, resemble bacteria in their metabolic processes but have similarities to eukaryotes in their apparatuses for information processing (DNA replication, transcription, and translation), although they have small circular genomes, like bacteria, suggesting that they diverged from bacteria early on.

Among the archaea whose replication origins have been characterized to date, most have multiple origins, with several components of the replication machinery conserved between archaea and eukaryotes. These include the proteins Orc1/Cdc6, MCM, and GINS (reviewed in reference 1). Thus, organisms of these two domains (*Archaea* and *Eukaryota*) appear to have evolved from a common ancestor, with the archaeal machinery being a fundamental version of what exists in eukaryotes. While eukaryotic ORC is a complex of six proteins and mediates the loading of Cdc6 and other proteins, in archaea, a single gene encodes a protein that shares sequence similarity with both eukaryotic Orc1 and Cdc6 (and is therefore referred to as Orc1/Cdc6). The eukaryotic MCM complex is a heterohexamer of MCM2-7 subunits, but the archaeal MCM complex is usually a homohexamer. The GINS complex in eukaryotes is a heterotetramer, while in archaea it is either a dimer of dimers or a homotetramer. The relatively simple organization of the archaeal replication machinery makes these organ-

isms suitable models for studying the functions of the individual components of the apparatus (reviewed in reference 2).

The archaeal Orc1/Cdc6 was first identified in *Pyrococcus furiosus* (3, 4). The numbers of Orc1/Cdc6 proteins vary among archaeal species, from 1 in *Pyrococcus* species and *Pyrobaculum aerophilum* (4) to more than 10 in some *Halobacterium* species (5). Most archaea possess one to three Orc1/Cdc6 orthologs (6–9). Almost every archaeal origin identified thus far adjoins an Orc1/Cdc6 gene. Studies using chromatin immunoprecipitation (ChIP) followed by analysis of the immunoprecipitated DNA on a whole-genome array chip (ChIP-chip studies) have revealed that the *oriC* is virtually the only site at which *Pyrococcus abyssi* Orc1/Cdc6 binds the genome (10). Recent work in *Sulfolobus islandicus* (11) has shown that two of its three origins are Orc1/Cdc6 dependent. Apart from origin recognition, Orc1/Cdc6 mediates the loading of the replicative helicase (MCM complex) onto DNA (12, 13).

The thermoacidophilic euryarchaeon *Picrophilus torridus*, which thrives at 55 to 60°C and pH 0.7, was isolated from a dry solfataric field in northern Japan (14). Whole-genome sequence analysis of *P. torridus* (15) suggests it has a typical archaeal replication apparatus, with a single Orc1/Cdc6 protein (PtOrc1/Cdc6), a single MCM, and a single-component GINS. This report pres-

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ents the results of the first efforts toward investigating replication events in *P. torridus*. The recombinant MBP (maltose binding protein)-tagged Orc1/Cdc6 protein has been characterized. Our results suggest a possible role for proliferating cell nuclear antigen (PCNA) in regulating replication events through its interaction with Orc1/Cdc6.

MATERIALS AND METHODS

***P. torridus* cultures.** *Picrophilus torridus* DSM 9790 (NBRC, Japan) was cultivated aerobically in liquid medium (0.2% yeast extract, 0.3% potassium dihydrogen phosphate, 0.05% magnesium sulfate, 0.025% calcium chloride, 0.02% ammonium sulfate, 1% glucose, pH 1.0) at 55°C and 100 rpm.

Cloning of *P. torridus* Orc1/Cdc6. The *Orc1/Cdc6* gene was amplified from *P. torridus* genomic DNA (isolated as described in reference 16 and the supplemental material), using Phusion DNA polymerase (Thermo Scientific, USA) and primers PtOrc1-F and PtOrc1-R (5'-GGATCCATG GACAATCCCTTTATT-3' and 5'-GTGACTCCTATATCATCATAAT TTGT-3', respectively), which were designed based on the sequence of the putative Orc1/Cdc6 annotated in the genome (15). The amplicon was cloned into pUC19 (NEB Inc., USA), and sequencing confirmed the authenticity of the clone.

To express the Orc1/Cdc6 protein in *Escherichia coli*, the *Orc1/Cdc6* gene was subcloned into the BamHI-SalI sites of pASK-IBA43plus (IBA BioTAGnology, Germany), creating plasmid pASK-Orc1/Cdc6. pASK-Orc1/Cdc6ΔN-term was created by digesting pASK-Orc1/Cdc6 with SacI and ligating together the ends thus generated, while pASK-Orc1/Cdc6ΔC-term was created by digesting pASK-Orc1/Cdc6 with HindIII and ligating together the ends thus generated. *Orc1/Cdc6* was subcloned into the BamHI-SalI sites of pMAL-c2x (NEB Inc., USA) using primers PtOrc1-F and PtOrc1-R2 (5'-GTCGACTTATATATCATCATAATTTGT CCT-3'), creating plasmid pMAL-Orc1/Cdc6.

Purification of *Picrophilus* Orc1/Cdc6 and raising antibodies. Orc1/Cdc6ΔN-term and MBP-Orc1/Cdc6 proteins were expressed in *E. coli* and purified as described in the supplemental material. Polyclonal antibodies against purified recombinant Orc1/Cdc6ΔN-term protein were raised in mice. Three mice were immunized with 50 μg of purified protein using Freund's complete adjuvant. This was followed by five booster doses with the same amount of protein using the incomplete adjuvant at 10-day intervals. The mice were bled 10 days after the fifth booster.

Cloning of *P. torridus* PCNA. The PCNA gene was amplified from *P. torridus* genomic DNA using primers PtPCNA-F and PtPCNA-R (5'-CA CCGAATTCATGACAAGGATGAGTATATCTG-3' and 5'-TATCTCGA GAGGATGATTCCATCCTTGGGGC-3', respectively), which were designed based on the sequence of the putative PCNA annotated in the genome. The amplicon was cloned into pUC19 (NEB Inc., USA) and confirmed to be PCNA by sequencing. PCNA was subcloned into the EcoRI-PstI sites of pASK-IBA43plus, creating plasmid pASK-PCNA. The recombinant PCNA expressed from this clone was tagged with six histidine residues at its N terminus. The recombinant protein was purified as described in the supplemental material.

ATP hydrolysis assay. The recombinant MBP-Orc1/Cdc6 (and MBP-Orc1/Cdc6-K67A) was assessed for ATP hydrolysis activity. MBP-Orc1/Cdc6 protein eluted from the amylose resin was further subjected to gel filtration chromatography on Superdex 200, and the fractions carrying the protein were identified by absorbance at 280 nm, followed by SDS-PAGE. The protein obtained after gel filtration chromatography was incubated with 10 to 400 μM ATP in a 10-μl reaction mixture containing 50 mM HEPES (pH 7.4), 5 mM MgCl₂, 1.2 mM beta-mercaptoethanol, 100 mM KCl, and [γ -³²P]ATP (0.5 μCi) as a tracer, for 60 min at 55°C before stopping the reaction by the addition of 50 mM EDTA. Reactions with DNA had 1 μM DNA. One- to 3-μl aliquots of the reaction mixtures were resolved by thin-layer chromatography using polyethyleneimine (PEI)-cellulose sheets (Merck, USA). The chromatogram was developed in 0.6 M Na₂HPO₄/NaH₂PO₄ (pH 3.5), air dried, and subjected to phosphorim-

aging (FLA-5000; Fuji Phosphorimaging System). The fraction of input ATP that was hydrolyzed was determined on the basis of the hydrolyzed proportion of the radioactively labeled ATP. This was then multiplied by the total amount of ATP in the reaction mixture to determine the amount of ATP hydrolyzed. Reaction mixtures containing buffer in place of protein were used as negative controls, and all values reported here were obtained after deducting corresponding values of the negative controls. Experiments were performed three times, and mean values are represented by bars, with error bars indicating standard deviations.

DNA binding reactions of Orc1/Cdc6. Electrophoretic mobility shift assays (EMSAs) were used to assess Orc1/Cdc6 interactions with DNA. A radiolabeled double-stranded oligonucleotide carrying an ORB (origin recognition box) consensus sequence was used as the substrate. For *Sulfolobus*- and *Pyrococcus*-derived ORB sequences, the sequence of the upper strand was 5'-GTTCCAGTGGAAACGAAAGGGGG-3' (conserved residues are in boldface and underlined), and that of the lower strand was 5'-CCCCCTTTCGTTTCCACTGGAAC-3'. For *P. torridus*-derived ORB sequences, the sequence of the upper strand was 5'-TATCATTTTCCAAT GGAAGCATTGAATA-3', and that of the lower strand was 5'-TATTC AATGCTTTCCATTGGAAATGATA-3'. EMSA was also performed using a radiolabeled double-stranded oligonucleotide with an arbitrary sequence whose upper-strand sequence was 5'-CATGACATCGCTAACT TGACCTATCAGCGGCACTATTA-3'. Protein (1,500 nM) was incubated with 0.2 pmol of γ -³²P-labeled DNA substrate (13.5 nM in a 15-μl reaction mixture) in a buffer containing 50 mM Tris-Cl (pH 8.0), 20 mM NaCl, 10 mM MgCl₂, 0.4 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol on ice for 15 min (reaction mixtures were also incubated at 22, 37, 50, and 55°C, and binding was more or less equivalent at all temperatures tested). Reactions were stopped by adding dye (bromophenol blue in 50% glycerol) and resolved by polyacrylamide native gel electrophoresis (6%) in 1× Tris-borate-EDTA (TBE) buffer (run conditions, 30 mA, 2 h, 16°C). The results were analyzed by autoradiography. Chase was done using an excess of the ORB-containing double-stranded DNA (ds-DNA) oligonucleotide substrate of the upper-strand sequence 5'-GTTC CAGTGGAAACGAAAGGGGG-3' or 5'-TATCATTTCCAATGGAAAG CATTGAATA-3' (up to 50-fold excess) and with a double-stranded DNA oligonucleotide substrate with an arbitrary sequence (upper-strand sequence, 5'-CATGACATCGCTAACTTGACCTATCAGCGGCACTATT A-3'; up to 100-fold excess).

Preparation of *P. torridus* extracts. For analysis of Orc1/Cdc6 expression in *P. torridus*, cells were harvested from 50-ml cultures at an optical density at 600 nm (OD₆₀₀) of ~0.4, and cell pellets were directly resuspended and boiled in SDS-PAGE sample loading buffer. One-tenth was resolved by SDS-PAGE for Western blot analysis using anti-Orc1/Cdc6 antibodies. For immunoprecipitations, *P. torridus* whole-cell extracts were isolated from cells of 50- to 100-ml cultures grown to an OD₆₀₀ of ~0.4. The harvested cells were washed with 1× phosphate-buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate, pH 7.4) and then resuspended in 1× PBS containing protease inhibitors. The cell suspension was sonicated, and cell debris was removed by high-speed centrifugation (16,000 × g) to obtain clarified whole-cell lysates.

Immunoprecipitations and pulldowns. PCNA was immunoprecipitated from whole-cell extracts of cells grown in 100-ml cultures to an OD₆₀₀ of ~0.4. A protein A Sepharose/CL6B Sepharose slurry mixture (1:1; Sigma-Aldrich, USA) was equilibrated with 1× PBS and incubated with 5 μl mouse anti-PCNA antibody (raised in the laboratory) at 4°C, with intermittent mixing every 5 min for 1.5 h. The unbound antibodies were removed by washing the beads extensively with 1× PBS containing 0.2% Triton X-100. The bead-bound antibodies were incubated with *P. torridus* whole-cell lysate overnight at 4°C using a nutator mixer. After extensive washes to remove the unbound proteins, the beads were boiled in SDS sample buffer, and the bound fraction thus released was resolved on SDS-PAGE, followed by Western blotting. For Western blotting, reactions resolved by SDS-PAGE were transferred onto a polyvinylidene di-

fluoride (PVDF) membrane; the membrane was blocked with 5% bovine serum albumin (BSA) in 1× PBS and then incubated with primary antibodies (anti-PCNA at 1:5,000 dilution and anti-Orc1/Cdc6 at 1:1,000 dilution) for an hour at room temperature. After extensive washing, the membrane was incubated with the horseradish peroxidase (HRP)-labeled secondary antibody for half an hour at room temperature before developing the blot by addition of suitable substrate. Detection was by the chemiluminescence method.

In pull-down assays, purified PCNA and purified (wild-type or mutant) MBP-Orc1/Cdc6 proteins (the purification procedures are described in the supplemental material) were incubated in 500 μ l 1× PBS with mixing for 2 h at 4°C using a nutator mixer. PCNA (and any interacting MBP-Orc1/Cdc6) was then pulled down from the mixture using cobalt affinity beads (BD Biosciences) by incubating the mixture with the beads equilibrated with 1× PBS for 30 min at 4°C. After removing the unbound fraction, the beads were washed extensively with 100 mM Tris-Cl (pH 8.0), 1 M NaCl, and the bound fraction was eluted with 100 mM Tris-Cl (pH 8.0), 300 mM NaCl, 250 mM imidazole. The eluate fractions were resolved on SDS-PAGE and analyzed by Coomassie staining.

Gel filtration analysis. Gel filtration analysis was carried out on a Superdex 200 10/300 GL analytical column (GE Healthcare Life Sciences) using the AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences). Proteins were resolved chromatographically in 50 mM potassium phosphate (pH 7.4), 150 mM NaCl. The column was calibrated by chromatographically resolving protein standards of known molecular masses (carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; β -amylase, 200 kDa; obtained from Sigma-Aldrich). Four hundred micrograms of MBP-Orc1/Cdc6 proteins (wild type or mutant) was loaded in individual protein analyses. To analyze any possible interactions between MBP-Orc1/Cdc6 (wild type or mutant) and PCNA, 280 μ g of MBP-Orc1/Cdc6 protein was incubated with 280 μ g PCNA in 50 mM potassium phosphate (pH 7.4), 150 mM NaCl for 2 h with mixing at 4°C before loading the mixture onto the column. The molecular masses of MBP-Orc1/Cdc6 and PCNA proteins were estimated from their retention volumes, using those of the standards.

RESULTS

PtOrc1/Cdc6 possesses the motifs conserved in other archaeal Orc1/Cdc6 members. The extremophile euryarchaeon *P. torridus* thrives at pH 0 to 1 and has an intracellular pH of $\sim$ 4.6. Based on analysis of its whole-genome sequence (15), it is predicted to have a typical archaeal replication apparatus. *P. torridus* shares features with both the euryarchaeon *Pyrococcus* and the crenarchaeon *Pyrobaculum aerophilum* (4, 10) in that it has only one *Orc1/Cdc6* gene (PTO1257; KEGG database). The *PtOrc1/Cdc6* gene was cloned by PCR amplification using genomic DNA as the template. The amplicon obtained was of the expected size of $\sim$ 1.2 kb, and its sequence matched that of PTO1257.

A comparison of the PtOrc1/Cdc6 amino acid sequence with Orc1/Cdc6 orthologs of other acidophiles, such as *Sulfolobus* species (with three Orc1/Cdc6 orthologs) (17, 18) and *Thermoplasma acidophilum* (with two Orc1/Cdc6 orthologs) (19, 20), revealed that PtOrc1/Cdc6 shares 25 to 55% identity and 43 to 74% similarity with these organisms (ClustalW analysis is shown in Fig. S1 in the supplemental material). It shares the highest similarity with Orc1/Cdc6-2 of *T. acidophilum* (55% identity and 74% similarity; [20]). PtOrc1/Cdc6 was found to possess the motifs typifying other archaeal Orc1/Cdc6 members (Fig. 1A). The Orc1/Cdc6 proteins are multidomain proteins containing an N-terminal AAA⁺ (ATPase associated with various activities) domain and a C-terminal winged-helix (wH) DNA binding domain. PtOrc1/Cdc6 has a similar architecture. The N-terminal AAA⁺ ATPase

FIG 1 (A) Conserved domains in PtOrc1/Cdc6. (B) Western blot analysis of recombinant PtOrc1/Cdc6 with anti-Orc1/Cdc6 antibody (1:1,000). The arrow indicates PtOrc1/Cdc6. (C) Western blot analysis of 50 μ g *P. torridus* whole-cell extracts using anti-Orc1/Cdc6 antibodies (1:1,000).

domain (residues 37 to 196) harbors the Walker A and Walker B motifs (residues 61 to 68 and 142 to 147, respectively) (Fig. 1A; see Fig. S1 in the supplemental material) that are responsible for ATP binding and hydrolysis.

The Orc1/Cdc6 protein is expressed in *P. torridus*. To examine Orc1/Cdc6 expression in *P. torridus*, we adopted the route of raising antibodies against the purified recombinant protein to be used to analyze *P. torridus* extracts. Accordingly, the *Orc1/Cdc6* gene was subcloned into pASK-IBA43plus. However, the Orc1/Cdc6 protein expressed from this clone in *E. coli* was insoluble at both 37°C and 16°C. One possible reason for this could be the existence of essential posttranslational modifications in the native PtOrc1/Cdc6, the absence of which (in the recombinant protein) could negatively impact expression of the protein in soluble form in *E. coli*. The N- and C-terminal fragments of Orc1/Cdc6 were then expressed in *E. coli*, and while the Orc1/Cdc6 N-terminal fragment (amino acid residues 1 to 165) was insoluble, the C-terminal fragment (amino acid residues 209 to 408) was soluble at 16°C. Orc1/Cdc6 Δ N-term (the C-terminal fragment of Orc1/Cdc6) was purified by affinity-based chromatography (see Fig. S2A in the supplemental material). Polyclonal antibodies were raised against purified Orc1/Cdc6 Δ N-term, and the sensitivity of the antibodies was examined by Western blotting of recombinant Orc1/Cdc6. The antibodies were found to detect 5 ng of recombinant protein at a 1:1,000 antibody dilution (Fig. 1B), and when these antibodies were used to probe whole-cell extracts of *P. torridus*, the expected protein of $\sim$ 45 kDa was the major band detected (Fig. 1C). It is difficult to predict whether the protein is modified posttranslationally, as no anomaly in electrophoretic mobility is apparent.

DNA binding properties of Orc1/Cdc6. Archaeal origins contain short repeat sequences called ORBs, first identified as 36-bp repeats in *Methanobacterium* and *Pyrococcus* species (21). The

ORBs contain a conserved 13-bp core sequence called the mini-ORB (5'-t/cTNCANNNGAA/c-3'). ORBs have been identified in *Sulfolobus* species, *Pyrococcus* species, *Aeropyrum pernix*, *Archaeoglobus fulgidus*, *Halobacterium* species, and *Methanothermobacter thermautotrophicus*, and Orc1/Cdc6 has been found to bind specifically to these ORBs (17–27).

To investigate the ability of MBP-tagged PtOrc1/Cdc6 to interact with DNA, we initiated binding studies using an ORB consensus sequence derived from *Pyrococcus* and *Sulfolobus* species to design a suitable oligonucleotide substrate. The full-length recombinant Orc1/Cdc6 protein expressed from the pASK vector was insoluble, and all attempts to solubilize the protein failed, as in every case it precipitated out during the refolding process. We therefore cloned the Orc1/Cdc6 gene into the pMAL vector to improve the solubility of the recombinant protein. The expressed protein carried the MBP tag at its N terminus. MBP-Orc1/Cdc6 was ~30% soluble when expressed at 16°C and was purified to >98% homogeneity by affinity-based chromatography using amylose resin (Fig. 2A). MBP to be used as a control was also purified. The DNA binding properties of PtOrc1/Cdc6 were examined in electrophoretic mobility shift assays, using MBP-Orc1/Cdc6 and DNA substrate carrying a consensus ORB sequence from *Pyrococcus* and *Sulfolobus* species (22) (see Materials and Methods). We found that while MBP itself did not bind to the oligonucleotide substrate, MBP-Orc1/Cdc6 formed a complex with the DNA (Fig. 2B, left), indicating that the complex was due to PtOrc1/Cdc6-DNA interaction. We also analyzed the ability of MBP-Orc1/Cdc6 to bind to dsDNA with an arbitrary sequence and found that it was unable to do so (Fig. 2B, right). The interaction of MBP-Orc1/Cdc6 with the double-stranded ORB-containing DNA substrate was found to be almost completely competed out by 10-fold excess of the same substrate (cold chase). In contrast, the same interaction was only partially competed out by a 50-fold excess of cold DNA substrate with an arbitrary sequence (Fig. 2C).

ORB sequences are typically found upstream of Orc1/Cdc6 genes. We examined the DNA sequence upstream of PtOrc1/Cdc6 for ORB sequences and found several possible candidate DNA segments. Of these, two were found to contain mini-ORB sequences that matched those identified in other archaeal genomes perfectly. They are located approximately 2.1 kb and 1.85 kb upstream of the Orc1/Cdc6 gene. To ascertain if PtOrc1/Cdc6 binds to these putative Pt-ORB sequences *in vitro*, electrophoretic mobility shift assays were carried out using a Pt-ORB-derived DNA substrate (see Materials and Methods for the sequence). We found PtOrc1/Cdc6 to preferentially bind this substrate, with the protein-DNA complex competed out almost completely by a 10-fold excess of cold-specific substrate; in contrast, the complex was only partially competed out by a 50-fold and 100-fold excess of cold DNA substrate with an arbitrary sequence (Fig. 2D). Using the putative Pt-ORB DNA substrate, when the DNA binding properties of MBP-Orc1/Cdc6 were examined in the presence of ATP and its nonhydrolyzable analog ATP γ S, no significant difference in the binding of protein to dsDNA was apparent, indicating that ATP is not essential for DNA binding (Fig. 2E). MBP-Orc1/Cdc6 did not bind to single-stranded DNA, as was apparent from DNA binding reactions carried out using the two single strands of the Pt-ORB dsDNA oligonucleotide as the substrate (data not shown). More detailed experimentation needs to be carried out to ascertain whether these identified ORBs are bona fide ORB sequences that bind Orc1/Cdc6 *in vivo*.

ATP hydrolysis by PtOrc1/Cdc6 is mediated by its Walker A motif. The binding and hydrolysis of ATP by the initiator proteins DnaA and ORC are key events regulating the initiation of DNA replication in bacteria and eukaryotes, respectively (28–30). We analyzed the ability of PtOrc1/Cdc6 to hydrolyze ATP using MBP-tagged Orc1/Cdc6. For this purpose, MBP-Orc1/Cdc6 purified by amylose resin-based affinity chromatography was further subjected to gel filtration chromatography to remove any possible contaminant ATPases (as described in Materials and Methods) (see Fig. S2B in the supplemental material). The ATPase activity of 10 to 1,400 nM MBP-Orc1/Cdc6 protein was assessed, and about 400 pmol of the input 1,000 pmol ATP was hydrolyzed by 1,000 nM protein (Fig. 3A), in contrast to MBP alone, which displayed background levels of activity (data not shown). Thus, MBP-Orc1/Cdc6 displays moderate ATPase activity.

We found *P. torridus* Orc1/Cdc6-mediated ATP hydrolysis to be DNA independent (Fig. 3A). However, the addition of a double-stranded DNA oligonucleotide harboring ORB sequence had an inhibitory effect on ATP hydrolysis. This was seen over a range of ATP concentrations (10 to 400 μ M) (Fig. 3B). There was no effect on the addition of a dsDNA oligonucleotide with an arbitrary sequence (data not shown). ATP hydrolysis in AAA⁺ family members is mediated by the Walker domains (Walker A and Walker B). We created a mutation in the Walker A domain of Orc1/Cdc6 (Fig. 1A; see Fig. S1 in the supplemental material) and purified the MBP-tagged Orc1/Cdc6-K67A protein to >95% homogeneity (see Fig. S2C in the supplemental material). Its ability to hydrolyze ATP was compared with that of wild-type MBP-Orc1/Cdc6 (Fig. 3C). As is apparent from the data, MBP-Orc1/Cdc6 ATPase activity was severely compromised by mutating the essential lysine residue of the Walker A motif. These results confirm that the MBP-PtOrc1/Cdc6-ATPase activity is mediated by its Walker A domain.

Orc1/Cdc6 exists in a complex with PCNA in *P. torridus* extracts. While the ORC proteins play a central role in origin recognition and activation, the clamp protein PCNA plays an important role in modulating DNA synthesis by increasing the processivity of the replicative polymerases (31–33), as well as by recruiting several replication factors (reviewed in reference 34). We examined the possibility of PtOrc1/Cdc6 interacting with PCNA in immunoprecipitation reactions carried out with anti-PCNA antibodies (raised in the laboratory against recombinant PtPCNA). *P. torridus* whole-cell lysates were isolated, and PCNA was immunoprecipitated from them. Analysis of the immunoprecipitates by Western blotting using the anti-PtOrc1/Cdc6 antibodies revealed that PtOrc1/Cdc6 coimmunoprecipitated with PCNA (Fig. 4A). As the PtOrc1/Cdc6 antibodies we raised were of low titer, we did not find them suitable for carrying out immunoprecipitations to look for PtOrc1/Cdc6-interacting partners. The results in Fig. 4A strongly suggest that PtOrc1/Cdc6 exists in a complex with PCNA *in vivo*. The binding we detected between PCNA and PtOrc1/Cdc6 under the immunoprecipitation conditions was not in a 1:1 stoichiometry. This could be partly attributed to the low sensitivity of the PtOrc1/Cdc6 antibodies. More importantly, it may be because PCNA is known to interact with several proteins, some simultaneously and some sequentially, and thus, not all of the cellular PCNA would be interacting with PtOrc1/Cdc6 at a given time.

Orc1/Cdc6 interacts with PCNA directly. We addressed the

issue of whether the Orc1/Cdc6-PCNA interaction was direct or was mediated by one or more bridging proteins by performing direct pulldown experiments between the two purified recombinant proteins. MBP, MBP-Orc1/Cdc6, and PCNA were purified (Fig. 4B, lanes 1 to 3; described in the supplemental material), and PCNA was then incubated with either MBP or MBP-Orc1/Cdc6 in $1\times$ PBS; this was followed by immobilization of the PCNA on

cobalt affinity beads. After extensive washes to remove the unbound fraction, PCNA (and any interacting MBP/MBP-Orc1/Cdc6) was eluted using imidazole. The eluates were analyzed by resolution on SDS-PAGE, followed by Coomassie staining. As seen in [Fig. 4B](#) (lanes 4 to 8), while recombinant MBP-Orc1/Cdc6 by itself did not bind to the cobalt affinity beads (lane 7), MBP-Orc1/Cdc6 was pulled down, along with PCNA, which bound to

FIG 3 ATP hydrolysis assay. (A) ATP hydrolyzed as a function of the Orc1/Cdc6 concentration. Zero to 1,400 nM MBP-Orc1/Cdc6 was incubated with 100 μM ATP in the absence of DNA. (B) ATP hydrolyzed as a function of the ATP concentration. MBP-Orc1/Cdc6 (1,000 nM) was incubated with 10 to 400 μM ATP in the absence or presence of 1 μM DNA. (C) ATP hydrolysis by MBP-Orc1/Cdc6-K67A in comparison with wild-type MBP-Orc1/Cdc6. MBP-Orc1/Cdc6-K67A (or wild type MBP-Orc1/Cdc6) (1,000 nM) was incubated with 10 μM and 100 μM ATP in the absence of DNA. WT, wild-type MBP-Orc1/Cdc6; Mut, MBP-Orc1/Cdc6-K67A.

the cobalt affinity beads (lane 8). MBP alone was not pulled down along with PCNA (lane 6), signifying that the interaction was between PtOrc1/Cdc6 and PCNA. This is the first evidence of a direct interaction between Orc1/Cdc6 and PCNA in archaea. This has not been reported in higher eukaryotes, either.

Orc1/Cdc6-PCNA interaction is mediated by a PIP box motif in Orc1/Cdc6. Numerous proteins that interact with PCNA are characterized by the possession of a PIP (PCNA-interacting protein) box domain (QXXL/M/IXXF/YF/Y, where X is any amino acid) (32) through which they bind to PCNA. PCNA engages several crucial components of the replication apparatus, binding these factors via the same interaction loop. As it is typically trimeric in nature, it can potentially associate with three factors concurrently, suggesting that while some of these interactions may be sequential, others may be simultaneous. Analysis of the PtOrc1/Cdc6 amino acid sequence revealed the presence of three candidate motifs that might serve as the PCNA-interacting domain (Fig. 5A). To ascertain if any of these was the PIP box mediating

FIG 4 (A) Immunoprecipitation analysis. PCNA immunoprecipitate was resolved by SDS-PAGE, followed by Western blot analysis with PCNA antibodies (1:5,000 dilution) or Orc1/Cdc6 antibodies (1:1,000 dilution). Lane 1, input lysate; lane 2, molecular mass marker; lane 3, immunoprecipitation reaction; lane 4, lysate control (an equal amount of *Picrophilus* lysate incubated with beads in the absence of antibody); lane 5, antibody (Ab) control (bead-bound PCNA antibodies; lysate not added). IgH and IgL, heavy and light chains of anti-PCNA antibodies used in IP. (B) Coomassie staining of SDS-PAGE analysis of a PCNA pull-down experiment. Lanes 1 to 3, input (purified) proteins; lanes 4 to 8, analysis of eluate fractions of pull-downs (lane 4, recombinant PCNA alone incubated with cobalt affinity beads; lane 5, MBP alone incubated with cobalt affinity beads; lane 6, MBP plus PCNA incubated with the beads; lane 7, MBP-Orc1/Cdc6 alone incubated with cobalt affinity beads; lane 8, MBP-Orc1/Cdc6 plus PCNA incubated with the beads); lane M, molecular mass marker.

PCNA binding, we created a panel of mutants, altering the conserved residues of all three motifs to alanine (described in the supplemental material) (Fig. 5A). The mutated Orc1/Cdc6 proteins were expressed and purified in fusion with the MBP tag (see Fig. S3A in the supplemental material). Circular dichroism (CD) scans were performed to check if the mutated proteins had undergone any gross structural alterations, and it was found that they were similar to the wild-type protein (see Fig. S3C in the supplemental material).

The mutated MBP-Orc1/Cdc6 proteins were incubated with PCNA to determine if they were able to associate with it. Using the same pull-down procedure described above, we found that while mutations in the PIP1 and PIP2 motifs (double mutants Q37A/L40A and Q373A/Y380A, respectively) did not in any way impede the MBP-Orc1/Cdc6-PCNA interaction, mutations in the PIP3 motif (triple mutant Q398A/L401A/Y405A) abolished the binding of MBP-Orc1/Cdc6 to PCNA (Fig. 5B). Thus, the motif QSK LRTNY (amino acid residues 398 to 405) serves as the PIP domain responsible for interacting with PCNA.

To analyze the importance of all three conserved residues of the motif QSKLRTNY in mediating the interaction with PCNA, each of the three underlined amino acids was individually mutated. CD scans of the purified proteins revealed that the mutated proteins

FIG 5 (A) Sequence of putative PIP boxes (PIP1 to -3) and mutations created. (B and C) Coomassie staining of SDS-PAGE analysis of pull-downs using PCNA and mutant Orc1/Cdc6 proteins. Lanes M, molecular mass marker; lanes 1, PCNA alone incubated with cobalt affinity beads; lanes 2, wild-type MBP-Orc1/Cdc6 alone incubated with cobalt affinity beads; lanes 3, MBP-Orc1/Cdc6 plus PCNA incubated with the beads; lanes 4, mutant MBP-Orc1/Cdc6 alone incubated with cobalt affinity beads; lanes 5, mutant MBP-Orc1/Cdc6 plus PCNA incubated with the beads. The mutants used are indicated below the gels. Top arrowhead in each panel, MBP-Orc1/Cdc6; bottom arrowhead in each panel, PCNA.

had not undergone any gross structural alterations (see Fig. S3B and C in the supplemental material). The proteins were analyzed for PCNA interactions in pull-down experiments, and it was found that all three residues were essential for Orc1/Cdc6 to bind to PCNA (Fig. 5C). Taken together, the experimental results in Fig. 4 and 5 suggest that Orc1/Cdc6 associates with PCNA *in vivo* and that this interaction may be a direct interaction between the two, with residues Q398, L401, and Y405 of Orc1/Cdc6 being critical for the interaction.

Examination of Orc1/Cdc6-PCNA interaction using gel filtration analyses. The MBP-Orc1/Cdc6-PCNA interaction was also examined using gel filtration chromatography. A Superdex 200 (10/300 GL) analytical column (GE Healthcare Life Sciences) was calibrated using protein standards of known molecular masses ranging between 29 and 200 kDa, and purified MBP-Orc1/Cdc6 (wild-type and mutant) proteins were resolved chromatographically in individual protein runs. The monomeric molecular mass of these proteins derived from their amino acid sequence is predicted to be about 85 kDa. We found that all the MBP-Orc1/Cdc6 proteins exist as monomers in solution (Fig. 6, curves 1 to 3; data for Q373A/Y380A is not shown). The purified recombinant His-tagged PCNA, with a predicted monomeric molecular mass of 29 kDa, was also resolved on the Superdex 200 column. The elution profile of the protein revealed that it exists as a trimer in solution, with the protein eluting at about the same retention volume as MBP-Orc1/Cdc6 (Fig. 6, curve 4). MBP-Orc1/Cdc6 and PCNA were incubated together in equimolar ratio (taking MBP-Orc1/Cdc6 to be a monomer and PCNA to be a trimer) in

the column running buffer and then resolved by Superdex 200 chromatography. We found that the mixture eluted as a single peak at a higher molecular mass than either MBP-Orc1/Cdc6 or PCNA (Fig. 6, curve 6). Based on the column calibration with the standards, the molecular mass of the peak corresponded to ~165 kDa. Thus, it appears that MBP-Orc1/Cdc6 and PCNA interact at a 1:1 ratio, despite the fact that PCNA is trimeric and therefore has three potential sites for MBP-Orc1/Cdc6 binding (SDS-PAGE analyses of peak fractions are shown for curve 1 [MBP-Orc1/Cdc6 only], curve 4 [PCNA only], and curve 6 [MBP-Orc1/Cdc6 plus PCNA]). The peak obtained was somewhat broad on one side, with the broad portion overlapping the MBP-Orc1/Cdc6 monomer/PCNA trimer peaks. This implies that not all of the two proteins were complexed with each other. The MBP-Orc1/Cdc6-Q37A/L40A and Q373A/Y380A proteins were similarly incubated with PCNA, and the mixtures were resolved by Superdex 200 chromatography, and in both cases, a single high-molecular-mass peak corresponding to the interaction of the MBP-Orc1/Cdc6 protein with PCNA at a 1:1 ratio was obtained (Q37A/L40A [Fig. 6, curve 7] and Q373A/Y380A [data not shown]). When MBP-Orc1/Cdc6-Q398A/L401A/Y405A was incubated with PCNA and the mixture was subjected to chromatography on Superdex 200, a single peak with a molecular mass of ~88 kDa was obtained (Fig. 6, curve 5). As the molecular masses of both native proteins (MBP-Orc1/Cdc6, monomer; PCNA, trimer) are 85 to 88 kDa, this result signified that the MBP-Orc1/Cdc6-Q398A/L401A/Y405A protein did not interact with PCNA. These data are in

FIG 6 Gel filtration analysis of MBP-Orc1/Cdc6 proteins. Curve 1, MBP-Orc1/Cdc6 (wild type) only; curve 2, MBP-Orc1/Cdc6-Q37A/L40A only; curve 3, MBP-Orc1/Cdc6-Q398A/L401A/Y405A only; curve 4, PCNA only; curve 5, MBP-Orc1/Cdc6-Q398A/L401A/Y405A plus PCNA; curve 6, MBP-Orc1/Cdc6 (wild type) plus PCNA; curve 7, MBP-Orc1/Cdc6-Q37A/L40A plus PCNA. The elution profile of protein standards (in kDa) is indicated by the arrows.

keeping with the results obtained in the pulldown experiments described above.

PCNA stimulates MBP-Orc1/Cdc6-mediated ATP hydrolysis. To uncover any role PCNA may play in modulating Orc1/Cdc6 activity, we examined MBP-Orc1/Cdc6-mediated ATP hydrolysis in the presence of PCNA. MBP-Orc1/Cdc6 (purified by successive chromatography on amylose resin and Superdex 200) was preincubated with PCNA for 2 min in the ATPase reaction buffer before the addition of ATP. We found that PCNA had no impact on ATP hydrolysis by MBP-Orc1/Cdc6 in the absence of DNA. However, when MBP-Orc1/Cdc6 was preincubated with PCNA for 2 min in the presence of ORB-containing DNA substrate and ATP was added later, we found that ATP hydrolysis was enhanced in comparison with MBP-Orc1/Cdc6-mediated ATP hydrolysis in the presence of ORB DNA alone (Fig. 7A). MBP-

FIG 7 (A) Effect of PCNA on ATP hydrolysis by PtOrc1/Cdc6. MBP-Orc1/Cdc6 (1,000 nM) and MBP-Orc1/Cdc6-Q398A/L401A/Y405A (1,200 nM) were assessed for ATP hydrolysis activity in the absence or presence of DNA, with or without preincubation of the proteins with PCNA. Reactions were carried out with 100 μ M ATP for 60 min. Orc1/Cdc6 and PCNA were mixed in a 1:1 (mole/mole) ratio, assuming Orc1/Cdc6 to be monomeric and PCNA to be trimeric. (B) Putative PIP boxes in Orc1/Cdc6 proteins from other archaea. Conserved residues are in boldface and underlined.

Orc1/Cdc6-Q398A-L401A-Y405A, which has lost its ability to interact with PCNA, was likewise analyzed. In this case, preincubating the mutant MBP-Orc1/Cdc6 protein with PCNA made no difference, as ATP hydrolysis values were similar with or without PCNA in the reaction mixture, regardless of the presence of ORB DNA (Fig. 7A). These results indicate that PCNA stimulates MBP-Orc1/Cdc6-mediated ATP hydrolysis in the presence of DNA substrate.

DISCUSSION

Although archaea are phylogenetically distinct from bacteria and eukaryotes, they resemble eukaryotes in their information-processing mechanisms. While the key events of bacterial and eukaryotic DNA replication have been thoroughly examined, less is known about archaea, largely due to the extreme conditions that many of them survive and multiply under, making their cultivation in the laboratory a challenge. Additionally, they are not easily manipulated. Typically, crenarchaeotes, like *Sulfolobus*, *Aeropyrum*, and *Pyrobaculum* species have multiple origins (24, 35–37). Euryarchaeota, however, have either one origin, like *Pyrococcus* and *Archaeoglobus* species (38, 39), or multiple origins, like *Methanocaldococcus* (39), *Halobacterium* (5, 40), and *Haloferax* (41) species. The euryarchaeon *P. torridus* is among the most acidophilic organisms isolated to date, growing at an optimum of pH 0.7. With a genome size of 1.55 Mb, it has a characteristic archaeal replication apparatus. Here, we have examined the Orc1/Cdc6 initiator protein.

PtOrc1/Cdc6 was found to carry a DNA binding module in the form of the winged helix and the AAA⁺ module that contains the Walker motifs essential to ATP hydrolysis (Fig. 1A). Archaeal Orc1/Cdc6 proteins have been found to bind to short consensus sequences within origins called ORBs (17–27). These ORBs carry a conserved core sequence. DNA-footprinting analyses of *Aeropyrum pernix* Orc1/Cdc6 has revealed that the binding of the protein to multiple ORB sites at the replication origin leads to distortion of DNA in this region (26). Of the multiple ORB and mini-ORB sites identified in the *P. furiosus* *oriC* region (10, 27), ChIP-chip analyses have revealed that Orc1/Cdc6 binds to two *in vivo* (10). The *Methanothermobacter thermautotrophicus* origin has an array of 13-bp ORB (MthORB) sequences, which are somewhat different from the ORBs of *Pyrococcus* and *Sulfolobus* species that are homologous to each other (9, 22). Orc1/Cdc6-1 from *M. thermautotrophicus* binds specifically to MthORB sequences and does not recognize ORBs of other archaea. We examined the DNA sequence upstream of PtOrc1/Cdc6 for ORB sequences, and of the several possible DNA segments that might serve as ORBs, two were found to carry mini-ORBs that perfectly matched those found in other archaeal genomes. They were between positions 1263151 to 1263162 and 1263442 to 1263453 of the genome.

On investigating the DNA binding properties of the MBP-tagged Orc1/Cdc6, we found that the protein binds to DNA carrying ORB sequences but not to DNA with an arbitrary sequence (Fig. 2B, C, and D). The bacterial initiator DnaA complexed with ATP forms active multimeric complexes with origin DNA (*oriC*) (28), and the interaction of ORC with origin DNA in eukaryotes is also dependent on ATP binding (29, 30). Though ORC mediates the hydrolysis of ATP, it is only ATP binding, and not its hydrolysis, that is essential to DNA binding (30, 42). The interaction of MBP-Orc1/Cdc6 with DNA was found to be independent of ATP (Fig. 2E). This has been reported to be the case in *Sulfolobus solfataricus* Orc1/Cdc6-1, as well (17). It is possible, however, that ATP alters the mode of interaction of Orc1/Cdc6 with origin DNA. Orc1/Cdc6 of *P. furiosus* binds to ORB sequences by different modes in the presence and absence of ATP (12). The crystal structure and biochemical analyses of the Orc1/Cdc6 proteins of other archaea have revealed that, although DNA binding is primarily mediated by the C-terminal winged-helix domain, the AAA⁺ module also interacts with and plays a role in restructuring origin DNA (23, 43–46).

The bacterial initiator DnaA and eukaryotic ORC and Cdc6 proteins are potent ATPases, and sequential hydrolysis of ATP by Cdc6 and ORC helps load the MCM2-7 complex (47). When MBP-Orc1/Cdc6 was analyzed for its ability to hydrolyze ATP, it was found to be a moderate ATPase. Though the ability of PtOrc1/Cdc6 to hydrolyze ATP was DNA independent, the ATPase activity was partially repressed by DNA containing ORB sequences. In this, PtOrc1/Cdc6 partly resembles Orc1/Cdc6 from *P. furiosus*, which, though displaying ATPase activity in the absence of DNA, exhibits strong suppression of this ATPase activity by DNA fragments carrying ORB sequences, but not by nonspecific DNA (12). The implications of this finding are unclear at this time, but it is possible that the interaction of Orc1/Cdc6 with its DNA substrate induces a conformational change in the protein that is unfavorable to ATPase activity. Orc1/Cdc6 of *S. solfataricus* has also been found to hydrolyze ATP (17). ATP binding/hydrolysis by PtOrc1/Cdc6 may play a role in effecting the subsequent association of other essential replication proteins, such as the MCM complex.

Several enzymes and other protein factors mediate the synthesis of the two strands of DNA, with DNA polymerases playing a major role. In eukaryotes, PCNA is responsible for “clamping” the replicative polymerases to the template DNA, and thus, it is responsible for increasing the processivity of these enzymes (31–33). Although PCNA was first identified as the DNA polymerase δ processivity factor, this trimeric ring around DNA not only anchors the DNA polymerases, but interacts with other replication-associated factors, as well (reviewed in reference 34). These are mainly components of the replication enzymatic machinery, such as replication factor C, replication protein A, Flap endonuclease I, and DNA ligase. The interaction of PCNA with its binding partners is mediated by the conserved PIP box motif in the interacting proteins, which bind to a specific site on PCNA (48). Since PCNA is trimeric, it can potentially interact simultaneously with three different proteins. In *Sulfolobus*, the distinct subunits of the heterotrimeric PCNA preferentially interact with specific partners, FEN1, DNA Pol B1, and DNA ligase I (49, 50). Thus, while PCNA itself lacks enzymatic activity, by simultaneously coordinating the activities of several other proteins, it is at the heart of the replication process. A detailed investigation of the archaeal DNA replication protein network by Li et al. (51) revealed that PCNA interacts with a number of proteins in *Thermococcus kodakaraensis*. Other than the expected candidates, such as DNA polymerase D, primase, RFC, and Fen1, some novel interacting partners were also detected, including Orc1/Cdc6, MCM1, and MCM2. However, as they were identified as proteins that copurify with PCNA from whole-cell lysates, the study did not establish a direct interaction with PCNA. We found Orc1/Cdc6 to exist in complex with PCNA in *P. torridus* extracts (Fig. 4A), and furthermore, we determined that Orc1/Cdc6 and PCNA interact directly (Fig. 4B). The site of this interaction in Orc1/Cdc6 was identified as a PIP box near the C terminus of the protein, and specific residues essential for the interaction were identified (Fig. 5). The interaction of Orc1/Cdc6 with PCNA was also evident in gel filtration studies, the results of which reaffirmed our finding that the interaction was mediated by the PIP box at the C terminus of the protein. While several studies have demonstrated a direct interaction between Orc1/Cdc6 and the MCM helicase (17, 19, 52–55), this study presents the first evidence of a direct interaction between Orc1/Cdc6 and PCNA. Sequence analyses of Orc1/Cdc6 from other archaea reveal that Orc1/Cdc6 of *P. torridus* is not the only archaeal Orc1/Cdc6 to harbor a PIP box motif (Fig. 7B). While the location of the PIP box in Orc1/Cdc6 is not conserved across the different species, the motif is present in several euryarchaeota members, such as *Halobacterium* and *Pyrococcus* species. Thus, Orc1/Cdc6 may potentially interact with PCNA directly in several archaea, which may share a mechanism of replication regulation.

The initiator proteins across all three domains of life are AAA⁺ ATPases, whose activities are regulated by the binding and/or hydrolysis of ATP. The *E. coli* initiator DnaA has high affinity for both ATP and ADP, but only the ATP-bound form of DnaA supports replication initiation *in vitro*. The DnaA-ATP complexes assemble into a multimeric complex at the origin and bring about the unwinding of DNA. Studies have unraveled mechanisms by which DnaA is active at the time of replication initiation but is subsequently inactivated to prevent reinitiation in the same cell cycle (reviewed in references 56 and 57). The regulatory inactivation of DnaA (RIDA) is brought about by ATP hydrolysis, which converts the active DnaA-ATP complex into inactive DnaA-ADP.

While the ADP-bound form can interact with origin DNA, it is unable to bring about the melting of the duplex (58, 59). DnaA itself does not display robust ATPase activity, but the hydrolysis of ATP is triggered by its interaction with two proteins: Hda (homologous-to-DnaA) and the functional ortholog of PCNA in bacteria, β -subunit of DNA Pol III (28, 60). Although the mechanism by which these proteins stimulate ATP hydrolysis of DnaA-ATP is not understood, RIDA is the main means of DnaA-ATP inactivation at the *oriC*. PtPCNA promotes PtOrc1/Cdc6-mediated ATP hydrolysis in a DNA-dependent manner. It is possible that a matching mechanism exists in archaea, where Orc1/Cdc6 binds origin DNA, activates origins in an ATP-dependent manner, and is then inactivated by the hydrolysis of bound ATP so that origins do not refire in the same cell cycle. A similar hypothesis has recently been proposed by others (61). Our findings underline the importance of studying DNA replication in archaea, as it uncovers diverse mechanisms of replication regulation in this largely conserved cellular process.

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