The GINS Complex from *Pyrococcus furiosus* Stimulates the MCM Helicase Activity*

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*Pyrococcus furiosus*, a hyperthermophilic Archaea, has homologs of the eukaryotic MCM (mini-chromosome maintenance) helicase and GINS complex. The MCM and GINS proteins are both essential factors to initiate DNA replication in eukaryotic cells. Many biochemical characterizations of the replication-related proteins have been reported, but it has not been proved that the homologs of each protein are also essential for replication in archaeal cells. Here, we demonstrated that the *P. furiosus* GINS complex interacts with *P. furiosus* MCM. A chromatin immunoprecipitation assay revealed that the GINS complex is detected preferentially at the oriC region on *Pyrococcus* chromosomal DNA during the exponential growth phase but not in the stationary phase. Furthermore, the GINS complex stimulates both the ATPase and DNA helicase activities of MCM in vitro. These results strongly suggest that the archaeal GINS is involved in both the initiation and elongation processes of DNA replication in *P. furiosus*, as observed in eukaryotic cells.

DNA replication is a fundamental event of life. Studies on the molecular mechanisms of DNA replication in a variety of organisms from the three biological domains, bacteria, Eukarya, and Archaea, are frequently published (for review, see Refs. 1–3). Among the phenomena associated with DNA replication, the regulation of DNA replication initiation is the most popular subject because the initiation of DNA replication is the major target for cell cycle control. The archaeal DNA replication machinery is most likely a simplified version of the eukaryotic system. Therefore, in addition to their intrinsic importance for understanding the evolution of the DNA replication apparatus, studies of archaeal DNA replication would help to elucidate the mechanisms common to both the archaeal and eukaryotic systems.

DNA replication begins with activation of the replication origin. In bacteria (eubacteria), the initiator protein, DnaA, binds to the single replication origin on the circular chromosome and recruits helicase to start replication. In contrast, in the eukaryotic replication system the replicative helicase is recruited to the origin recognition complex (ORC)³ at the origin by the functions of Cdc6 and Cdt1 to assemble the pre-replication complex during G1 phase. The replication helicase then starts to unwind the DNA bidirectionally from the origin to allow the replication forks to progress. Mini-chromosome maintenance (MCM) is a hexameric complex (Mcm2–7) that is similar to the bacterial DnaB helicase. The Mcm complex (MCM) is thought to be the core of the replicative helicase based on evidence that the Mcm4–6–7 subcomplex isolated from HeLa cell extracts displayed an oligonucleotide displacement activity (4). However, the Mcm2–7 purified from the extract lacks helicase activity in vitro, and thus, the activation of the MCM helicase is likely to involve posttranslational modification as well as associations with other factors. Actually, the MCM from a *Xenopus* egg extract exerted a weak helicase activity in the complex with the Cdc45 protein in a cyclin-dependent kinase-dependent manner (5). In addition, a yeast mutational analysis demonstrated that Cdc45 is required for the progression of replication forks (6). However, the machinery accomplishing the DNA unwinding function during the DNA replication process have not yet been clearly identified. Recently, some progress toward understanding the molecular machinery in more detail has been made with the proposal of a replisome progression complex. Immunoprecipitation experiments using *Xenopus* egg extracts detected a physical interaction between MCM, Cdc45, and the GINS complex proteins earlier (7). Recently, three reports demonstrated the isolation of replisome progression complexes including these three components from yeast (8) and *Drosophila* (9) as well as *Xenopus* egg extracts (10). The complex of Cdc45, MCM, and GINS is referred to as the CMG complex, after their isolation from yeast (9), or the “unwindosome” (10). The immunofluorescence-purified CMG complex displayed ATP-dependent unwinding activity of a 40-nucleotide oligonucleotide annealed to single-stranded DNA (9).

The archaeal genome sequences contain one to four genes encoding MCM homologs; however, many archaeal organisms have only one *mcm* gene, in contrast to eukaryotes (3). The MCM complexes from three archaeal organisms, *Methanothermobacter thermautotrophicus* (11–13), *Sulfurobacter solfataricus* (14, 15), *Archaeoglobus fulgidus* (16), and *Thermoplasma acidophilum* (17) have been characterized to date. In contrast to the heterohexameric eukaryotic MCM complex,
these archaeal MCMs are observed in various complex forms, including hexamer, heptamer, double-hexamer, and a filamentous form using a single subunit Mcm (11, 18–23). The archaeal MCMs have a distinct DNA helicase activity by themselves in vitro, and therefore, these MCMs may function as the replicative helicase in the archaeal cells. However, in our studies of the MCM helicase from Pyrococcus furiosus, a hyperthermophilic Euryarchaeae, we have detected only a very weak helicase activity in vitro from the protein.

The eukaryotic GINS complex consists of four different proteins, Sld5, Psf1, Psf2, and Psf3, originally identified in Saccharomyces cerevisiae as essential protein factors for the initiation of DNA replication (24). The amino acid sequences of the four subunits in the GINS complex share some conservation, suggesting that they are ancestral paralogs (25). Finely detailed bioinformatics analyses revealed the GINS protein homologs in archaeal genomes, although most of the Archaea have a single gene of this family (25). A sequence comparison showed that the crenarchaeal homologs are more similar to Psf2 and Psf3, and, in contrast, the euryarchaeal homologs are more similar to Sld5 and Psf1 (Ref. 25; also see Table 1 of this study). Furthermore, a GINS homolog, designated as Gins23, was detected in the Crenarchaeae, S. solfataricus, in a yeast two-hybrid screening for interaction partners of MCM, and another subunit, designated as Gins15, was identified by the mass spectroscopic analysis of an immunoaffinity-purified native GINS complex from S. solfataricus cell extract using an anti-GINS23 antibody (26). The S. solfataricus GINS complex, composed of two proteins, Gins23 and Gins15, forms a tetrameric structure with a 2:2 molar ratio (26).

In this study we characterized the complex of the GINS homologs from P. furiosus, a complex of Gins23 and Gins51 with a 2:2 ratio. This is the first report describing the euryarchaeal GINS. It is especially interesting that the DNA helicase activity of P. furiosus MCM is clearly stimulated by the GINS complex. The molecular mechanism of the MCM helicase stimulation by the GINS complex is discussed.

**EXPERIMENTAL PROCEDURES**

**Cloning of the mcm Gene**—The sequence encoding the mcm gene, corresponding to PF0482 in the data base, was amplified by PCR directly from P. furiosus genomic DNA. The PF0482 open reading frame contains an intervening sequence (an intein coded by 1110 nucleotides (~60 kDa)). Therefore, four primers were designed to fuse the two exteins via PCR to obtain the entire mcm gene. These primer sequences are available on request.

The DNAs encoding the N-terminal (1083 nucleotide) and C-terminal (963 nucleotide) exteins were amplified, and a second PCR was performed using the mixture of the two amplified fragments as DNA templates and the two primers corresponding to the N-terminal and C-terminal regions of the mcm gene. The amplified DNA containing the entire region of the mcm gene was inserted into the pGEM-T Easy vector (Promega), and the nucleotide sequence was confirmed. The cloned mcm gene was transferred into PET28a (Novagen) for expression in Escherichia coli cells (PET28a-MCM).

**Cloning of the Genes Encoding GINS Homologs**—The genes encoding the two GINS homologs, PF0483 (Gins23) and PF0982 (Gins51), were amplified by PCR directly from P. furiosus genomic DNA using the oligonucleotides 5'-GGCCGATAGCAGGATGTAATTGAT-3' and 5'-ACAGCCGGCGCCCTATACCTACC-3' as the forward and reverse primers for PF0483 and 5'-GGCCATATGGGATGACAAATGGATATTGA-3’ and 5’-CCCCGCGCCGTGTACAAATTGATACATACC-3’ as the forward and reverse primers for PF0982, respectively. The amplified genes were cloned into the pGEM-T Easy vector, and their nucleotide sequences were confirmed. The cloned genes were digested by NdeI-NotI and inserted into the corresponding sites of pET21a or pET28a (Novagen). The resultant plasmids were designated as pET21a-G23 and pET28a-G51, respectively.

**Overproduction and Purification of PfuMCM**—To obtain recombinant PfuMCM, E. coli BL21 CodonPlus™ (DE3)-RIL cells (Strategene) carrying pET28a-MCM were grown in 1.3 liters of LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. The cells were cultured to an A_600 of 0.50, and the expression of the gene encoding PfuMCM was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and continuing the culture for 4 h at 37 °C. After cultivation, the cells were harvested and disrupted by sonication in buffer A (20 mM phosphate buffer, pH 7.5, 0.5 M NaCl, and 0.5M NaCl, and 10% glycerol). The soluble cell extract, obtained by centrifugation at 12,000 × g for 10 min, was heated at 70 °C for 15 min. The heat-resistant fraction, obtained by centrifugation, was subjected to chromatography on a HiTrap™ Heparin HP column (GE Healthcare). The proteins that eluted at 0.2 m NaCl were dialyzed against buffer B (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 0.1 M NaCl and 10% glycerol). The dialyzed sample was loaded onto a HiTrap™ Heparin HP column (GE Healthcare), which was developed with a 0.1–0.8 M NaCl gradient in buffer B. The proteins that eluted at 0.35 M NaCl were dialyzed against buffer B. The dialyzed sample was loaded onto a Mono Q HR 5/5 column (GE Healthcare), which was developed with a 0.1–0.6 M NaCl gradient in buffer B. The proteins that eluted at 0.35 M NaCl were pooled and fractionated on a HiLoad™ 16/60 Superdex™ 200 column (GE Healthcare) equilibrated with buffer C (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 0.15 M NaCl, and 10% glycerol). The fractions that eluted at about 50 ml of retention volume were pooled and were stored at 4 °C.

**Overproduction and Purification of Gins23 and the PfuGINS Complex**—To obtain recombinant Gins23, E. coli BL21 CodonPlus™(DE3)-RIL cells (Strategene) carrying pET21a-G23 were grown at 37 °C in 1.3 liters of LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and 34 μg/ml chloramphenicol. The cells were cultured to an A_600 of 0.6, and the expression of the gene encoding Gins23 was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and continuing the culture for 3.5 h at 37 °C. After cultivation, the cells were harvested and disrupted by sonication in buffer D (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 0.5 M NaCl, and 10% glycerol). The soluble cell extract, obtained by centrifugation at 12,000 × g for 10 min, was heated at 80 °C for 25 min. The heat-resistant fraction, obtained by centrifugation, was treated with 0.15%
HisTrap™ HP column. The proteins that eluted at 0.2 M ammonium sulfate were precipitated by 80%-saturated ammonium sulfate. The precipitate was resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 0.5 M NaCl, and 10% glycerol). For the preparation of the PfuGINS complex, the recombinant E. coli strain producing the GINS complex was obtained by cotransformation of the E. coli BL21 CodonPlus™(DE3)-RIL cells with the two plasmids, pET21a-G23 and pET28a-G51. Both of the pET vectors have the colE1 ori and were incompatible for cotransformation in general. However, transformants containing both pET21a-G23 and pET28a-G51 were obtained by selection for AmpR and KanR colonies, although the efficiency was quite low. The transformed cells carrying pET21a-G23 and pET28a-G51 were grown in 1.3 liters of LB medium containing 50 μg/ml ampicillin and kanamycin and 34 μg/ml chloramphenicol at 37 °C. The cells were cultured to an A600 of 0.50, and the expression of the GINS genes was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM and continuing the culture for 4 h at 37 °C. After cultivation, the cells were harvested and disrupted by sonication in buffer A. The soluble cell extract, obtained by centrifugation at 12,000 × g for 10 min, was heated at 80 °C for 15 min. The heat-resistant fraction, obtained by centrifugation, was treated with 0.15% polyethyleneimine to remove the nucleic acids. The soluble proteins were precipitated by 80%-saturated ammonium sulfate. The precipitate was resuspended in buffer A and subjected to chromatography on a HiTrap™ HP column. The proteins that eluted at 0.2 M imidazole were dialyzed against buffer B (10 mM potassium phosphate, pH 6.8, 7 mM β-mercaptoethanol, 50 μM CaCl2, and 10% glycerol). The dialysate was loaded onto a hydroxyapatite column (CHT-II; Bio-Rad), which was developed instead of P. furiosus, obtained as described previously (28), were quickly cooled down to room temperature and then fixed with 1% formaldehyde for 15 min under aerobic conditions with gentle agitation. Samples for immunoprecipitation were prepared by sonication in an extraction buffer. Aliquots of the supernatant (0.5 ml) were incubated with 5 μl of anti-Cdc6, anti-MCM, and anti-Gins23 (prepared by using the purified Gins23 in this study), which were pretreated with protein A-Sepharose (GE Healthcare). The purified PfuMCM (500 nM) and PfuGINS proteins (various concentrations) were incubated with the 5'-32P-labeled substrate DNA (2.5 nM) in helicase buffer (25 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM DTT, 50 mM NaCl, 0.1 mg/ml bovine serum albumin, 3 mM ATP) for 30 min at 75 °C. After phenol extraction, the products were detected by 6% PAGE in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) followed by autoradiography.

**ATPase Assay**—The purified PfuMCM protein (500 nM) was incubated with 1 mM ATP containing [γ-32P]ATP (25 pCi/μl) in a buffer in the presence or absence of DNA (25 nM d49-mer, dAGCTGACTGCGAGTTGCGAATTAACCTGCTGTAATCAGGATGCTATAAGCAATTCGTAAATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTAC) and 10% glycerol. The molar extinction coefficients of tyrosine residues. The molar extinction coefficients of the Gins23 and PfuGINS components were calculated by measuring the absorbance at 280 nm. The theoretical molar absorption coefficients of the molecules were calculated based on the numbers of tryptophan and tyrosine residues. The molar extinction coefficients of Gins23 and PfuGINS are 31,720 and 38,120 M^-1 cm^-1, respectively.

**Gel Filtration and Glycerol Gradient Centrifugation**—Gel filtration chromatography for the estimation of the molecular sizes of the Gins23 and PfuGINS components was performed with the SMART system (Amersham Biosciences). The purified Gins23 and PfuGINS proteins were applied to a Superdex 200 3.2/30 column (Amersham Biosciences) pre-equilibrated with buffer C. The eluted fractions were subjected to SDS-PAGE. The molecular masses of the proteins were estimated from the elution profiles of standard marker proteins (Bio-Rad), including thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), and myoglobin (17,000). PfuGINS was also subjected to glycerol gradient centrifugation. The purified GINS complex (50 μg) was sedimented through a 10–35% (v/v) glycerol gradient at 40,000 rpm for 4 h in a Beckman SW41 rotor. The standard marker proteins, as described above, were sedimented under identical conditions.

**Helicase Assay**—For the preparation of the substrate for helicase assays, a 63-mer oligonucleotide (5'-TGCCAAGCTTGCGATGCCTGAGCTGACTCTAGAGATCCCCGGGTACCGAGCTCAGAATTCGTG3') was annealed to M13mp18 single-stranded DNA (Takara Bio) after labeling the 5' end with [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs). The partial DNA duplex was separated from the free oligonucleotides with a spin column (Microspin™ S-400 HR, GE Healthcare). The purified PfuMCM (500 nM) and PfuGINS proteins (various concentrations) were incubated with the 5'-32P-labeled substrate DNA (2.5 nM) in helicase buffer (25 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM DTT, 50 mM NaCl, 0.1 mg/ml bovine serum albumin, 3 mM ATP) for 30 min at 75 °C. After phenol extraction, the products were detected by 6% PAGE in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) followed by autoradiography.

**Chromatin Immunoprecipitation Assays**—The conventional chromatin immunoprecipitation assay procedure was performed as described previously (27). Pyrococcus abyssi cells, instead of P. furiosus, obtained as described previously (28), were quickly cooled down to room temperature and then fixed with 1% formaldehyde for 15 min under aerobic conditions with gentle agitation. Samples for immunoprecipitation were prepared by sonication in an extraction buffer. Aliquots of the supernatant (0.5 ml) were incubated with 5 μl of anti-Cdc6, anti-MCM, and anti-Gins23 (prepared by using the purified Gins23 in this study), which were pretreated with protein A-Sepharose (GE Healthcare) for 4 h with gentle shaking to collect the immunocomplexes. The precipitated DNA was extracted from the immunocomplexes as described (29). The obtained DNA was directly used as a template in PCR reactions with 1.25 units of TaqDNA polymerase (Takara Bio) for 20 cycles. Eleven different primer sets, corresponding to the region within 20 kb around the orIC of the P. abyssi genome were used (primer sequences are available on request; also see Fig. 4) with three different template dilutions to ensure the results of the assays. The intensities of each PCR product stained by ethidium bromide were quantified from digitized gel images, with the image analyzer (LAS-3000, Fuji Film) software accounting for differences in the amplification efficiency observed for different primer sets with the use of the input DNA. All results shown are the averages of three independent experiments.

**Western Blot Analysis**—P. furiosus cells (1 g) were disrupted by sonication in 15 ml of buffer B containing a proteinase inhibitor mixture (Complete™, Roche Applied Science), and the...
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extract was obtained by centrifugation. The _P. furiosus_ cell extract (800 ng) and the purified Gins23 protein (1 ng) were separated by 12% SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and reacted with the anti-Gins23 antiserum. The bands were detected by using an enhanced chemiluminescence system (GE Healthcare) according to the manufacturer’s instructions.

**Gel-retardation Assay**—Purified PfuMCM (100–800 nm) and PfuGINS proteins (100–2000 nm) were incubated with a 5′-32P-labeled deoxyoligonucleotide (d49-mer (see above)) or a duplex DNA with its complementary sequence in a binding buffer (20 mM Tris–HCl, pH 8.0, 1 mM MgCl2, 0.1 mg/ml bovine serum albumin, 0.5 mM ATP, and 25 mM DNA) at 60 °C for 10 min. The products were separated by 1% AGE (for GINS) in 1× TAE buffer and visualized by autoradiography.

**Two-hybrid Analysis**—A yeast two-hybrid detection system (MATCHMAKER GAL4 Two-Hybrid System 3, Clontech) was used for the detection of protein-protein interactions. The yeast strain AH109 was transformed with the appropriate plasmids (see the Fig. 3 legend) according to the manufacturer’s instructions (Clontech Matchmaker manual). The cells were streaked on either SD-Trp-Leu-His or SD-Trp-Leu-His-Adag agar plates and incubated at 30 °C.

**RESULTS**

**Conservation of the Genes for GINS in Archaea**—As shown in the previous report of the _S. solfataricus_ GINS proteins, one gene for each, encoding the sequences homologous to Gins23 and Gins15, was found in the _P. furiosus_ genome (26). The open reading frame numbers, PF0982 and PF0483, encode the homologs of Sld5/Psf1 and Psf2/Psf3, respectively. A comprehensive search for the GINS protein homologs in the archaeal genomes revealed that only thermococcal organisms in Eurarcheae, in contrast to all of the crenarchaeal genomes, except for *Thermofilum pendens*, have two genes encoding GINS homologs, as shown in Table 1. We cloned the two genes to characterize these gene products, which we designated as Gins51 (we prefer the name Gins51, rather than Gins15 because of the order G(5)-I(1)-N(2)-S(3)) and Gins23, respectively, as the _P. furiosus_ GINS components.

**Expression and Purification of the PfuMcm and PfuGINS Complex**—The His-tagged PfuMcm protein (M, 76805.57) was purified without difficulty by the process described under “Experimental Procedures” (Fig. 1A). A band corresponding to the same size as the recombinant PfuMcm was detected in the total cell extract of _P. furiosus_ by a Western blot analysis using anti-PfuMcm antiserum. A semiquantitative Western blot analysis showed that the _mcm_ gene was expressed constitutively from the early exponential phase to the late stationary phase (data not shown), as we previously reported for the _P. abyssi_ _mcm_ gene (27). The predicted genes for Gins51 and Gins23 encode proteins of 190 and 162 amino acids, with calculated masses of 21965.37 and 19201.58, respectively. The expression of the _gins23_ gene using the pET21a vector and the purification of the protein without any tag were performed in a straightforward manner. Starting from a 1-liter culture, 2.5 mg of homogeneous Gins23 protein were obtained, as shown in Fig. 1B.

| Euryarchaeae | Sld5/Psf1 | Psf2/Psf3 |
|-------------|-----------|-----------|
| Methanococcus maripaludis | MMP1710 | |
| Methanothermobacter jannaschii | MJ2048 | |
| Methanococcus mazei | MM1810 | |
| Methanosaeta thermophila | MM1810 | |
| Methanococcus horikoshii | PH0666 | PH0610 |
| Thermococcus kodakarenensis | TK0536 | TK1619 |
| Thermoplasma volcanii | TVN0553 | |
| T. acidophilum | Ta1042 | |
| Archaeoglobus fulgidus | AF1322 | |
| Methanopyrus kandleri | MK0416 | |

**Crenarchaeae**

| Euryarchaeae | Sld5/Psf1 | Psf2/Psf3 |
|-------------|-----------|-----------|
| Methanosaetaaceae | Hbut_0015 | Hbut_0904 |
| Aeropyrum pernix K1 | APE0437 | APE0187 |
| Pyrococcus horikoshii | PH0666 | PH0610 |
| Thermoplasma volcanii | TVN0553 | |
| T. acidophilum | Ta1042 | |
| Archaeoglobus fulgidus | AF1322 | |
| Methanopyrus kandleri | MK0416 | |

**FIGURE 1.** Production and purification of PfuMcm, Gins23, and the Pfu-GINS complex from _E. coli_ cells. A, purified His-tagged PfuMcm (0.1 μg) was separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). _P. furiosus_ cell extract (800 ng) and purified PfuMcm protein (recombinant, 1 ng) were separated by 10% SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and detected with anti-PfuMcm antiserum. B, purified Gins23 and PfuGINS complex (His-tagged at Gins51) proteins (0.4 μg each) were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. _P. furiosus_ cell extract (800 ng for each growth stage) and purified Gins23 protein (1 ng) were separated by 12% SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and detected with anti-Gins23 antiserum.

Furthermore, we detected a protein band with the same size as the recombinant Gins23 in cell extracts prepared from exponential and stationary phase _P. furiosus_ cell cultures (Fig. 1B). When the Gins51 protein was produced in the same manner, it was difficult to remove the nucleic acids from the protein. The
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**FIGURE 2. Oligomeric state of PfuGINS in solution.** A, gel filtration analyses of Gins23 and the PfuGINS complex. Each protein solution was fractionated on a Superdex 200 column. The elution profiles and the SDS-PAGE analyses of the peak fractions are shown. Elution of the proteins was monitored by the absorbance at 280 nm. The protein bands on the SDS-PAGE gels were detected by Coomassie Brilliant Blue staining. The oligomeric states of the PfuGINS complex and Gins23 were predicted by calculating the molecular weights from the retention times in each chromatography step. B, glycerol gradient centrifugation analysis. Purified PfuGINS was sedimented for 24 h at 4 °C through a 10–35% glycerol gradient. The standard curve was obtained from the retention times in each chromatography step.

**FIGURE 3. Protein-protein interactions shown by a yeast two-hybrid analysis and an immunoprecipitation analysis.** A, yeast AH109 cells were transformed with plasmids containing the DNA binding domain (DBD) or the activation domain (AD) fused to the open reading frame for Mcm, Gins23, or Gins51. V indicates empty vector. Results from two different incubation times (48 and 84 h) are shown. B, immunoprecipitation analyses were performed to confirm the formation of a complex including MCM and GINS in the P. furiosus cell extract. The immunocomplexes were captured with anti-MCM antiserum, as shown on the top, and were subjected to SDS-PAGE followed by Western blot analyses using anti-Gins23 antiserum. The extracts from the P. furiosus cells at the exponential phase (left panel) and the stationary phase (right panel) were analyzed. Precipitates without anti-MCM antiserum are loaded at the center (−) in each panel as negative controls.

Gins51 protein was coprecipitated when nucleic acids were precipitated by treatments with various concentrations of polyethyleneimine. Therefore, we tried to produce Gins51 as a complex with Gins23 by cotransformation of E. coli cells with the two plasmids encoding these genes. The two proteins were successfully produced in E. coli and migrated together during the purification procedure as described under "Experimental Procedures." The complex was finally purified to homogeneity, with equal stoichiometry (Fig. 1B). The Western blot analysis, showing constitutive production of Gins23 through the growth phases (Fig. 1B), suggests that the amount of GINS complex in the cells does not drastically vary throughout the pyrococcal cell cycle. To determine the molecular size of the complex in solution, the purified complex was subjected to gel filtration chromatography (Superdex 200), and a single clear peak containing both Gins23 and Gins51 with equal band intensities was obtained. From the elution position, the apparent molecular mass of the complex corresponded to 80,000, suggesting the formation of a complex of the two proteins with a two-to-two molar ratio (Fig. 2A). To confirm the molecular size of the complex in solution, a glycerol gradient separation experiment was performed. Two protein bands with equal intensities were detected at the position corresponding to the size of 78.8 kDa sedimented under these centrifugation conditions as shown in Fig. 2B). When only the Gins23 protein was subjected to gel filtration under the same conditions, a single peak was observed at the position corresponding to the dimer size. Because the Gins51 protein was not successfully purified by itself, it is not yet known whether Gins51 can form a stable homodimer. The dimer formation by Gins23 is in contrast to the case of the S. solfataricus GINS complex, in which Gins15, but not Gins23, forms a dimer (26). We designated the complex of P. furiosus Gins23 and Gins51 as PfuGINS.

**Physical Interactions of PfuGINS with PfuMCM and PfuOrc1/Cdc6—** The interactions of PfuGINS with other replication proteins were investigated by a yeast two-hybrid analysis. We inserted the genes encoding Gins51, Gins23, and Mcm as fusions with the gene encoding the GAL4 DNA binding domain or the activation domain in the plasmids for a two-hybrid system. As shown in Fig. 3A, the PfuGINS subunits interact with themselves (Gins51-Gins51 and Gins23-Gins23) and also with each other (Gins51-Gins23). This interaction mode is consistent with the complex formation with a two-to-two molar ratio of each subunit, as predicted from the results of the gel filtration and the glycerol gradient sedimentation analyses, as described above. In addition to the interactions between the Gins subunits, a clear positive signal was observed with the combination of Gins23 (but not Gins51) and Mcm (Fig. 3A). A physical interaction between Gins23 and Mcm by the two-hybrid system was also observed with the Sulfolobus proteins (26). The gene organization, in which the gins23 gene is located immediately upstream of the mcm gene on these archaeal genomes, is also conserved. To investigate the interaction between the GINS-MCM complexes in Pyrococcus cells, immunoprecipitation analysis was performed. As shown in Fig. 3B, Gins23 was
clearly detected in the immunocomplex obtained by coprecipitation with anti-PfuMcm antiserum from the extract of exponential phase cells. In contrast, the band intensity of Gins23 was decreased distinctly in extracts from stationary phase cells.

Our two-hybrid analysis also revealed that Gins51 interacts with Orc1/Cdc6, an origin-recognition protein in archaeal DNA replication (Fig. 3). Therefore, we investigated whether the archaeal GINS is localized in the oriC region during the growth of Pyrococcus cells by a chromatin immunoprecipitation assay. As shown in Fig. 4, the DNA fragment containing ORB1 (origin recognition box 1) and ORB2 (30) in the oriC region was specifically amplified from the chromatin fraction precipitated with the anti-Gins23 antiserum. The oriC region-specific amplification from the Gins23-bound chromatin DNA was observed only when the cell extract was isolated from Pyrococcus cells at the exponential growth phase but not from stationary phase cells. This is similar to the case when the chromatin is precipitated with anti-Mcm antiserum but is unlike the case when it is precipitated with anti-Orc1/Cdc6, since the Orc1/Cdc6 protein preferentially remains bound at the oriC region in both the exponential and stationary phases, as shown in our previous reports (27, 30). These results suggested that the PfuGINS complex functions as an initiation factor and also in fork progression with PfuMCM.

PfuGINS Stimulates the ATPase and Helicase Activities of PfuMCM—The recombinant PfuMCM protein has an ATP-dependent helicase activity (Fig. 5). However, the helicase activity is quite weak, as compared with that of the MCM proteins from other archaeal organisms, including M. thermautotrophicus (11–13) and S. solfataricus (14, 15). To investigate the effect of PfuGINS on the enzymatic activity of PfuMCM, the ATPase and helicase activities were compared in the presence and absence of PfuGINS in the reaction mixtures. As shown in Fig. 6, both the ATPase and helicase activities of PfuMCM were clearly stimulated by PfuGINS, whereas in contrast, the S. solfataricus GINS did not affect the helicase activity of SsoMcm (26). In P. furiosus cells, PfuMCM by itself may not be sufficient for the helicase activity, and thus, PfuGINS is required for PfuMCM to become a mature replicative helicase.

In our previous reports (27, 30), we demonstrated that PfuMCM preferentially binds to the oriC region of the Pyrococcus genome analyzed by the chromatin immunoprecipitation assay. Arrowheads indicate the ORB repeats. B, DNA binding by PfuGINS was quantified from the amount of PCR-amplified DNA by each primer set, and the results are shown as relative abundances based on the value at the 10-kb site (left side). The S.E. was obtained from three independent experiments. kb, kilobase(s).

FIGURE 5. PfuMCM has an ATP-dependent helicase activity. Purified PfuMCM was incubated with a partial DNA duplex with conditions described under “Experimental Procedures” in the presence or absence of ATP at 75 °C for 40 min. The reactions were monitored by 6% PAGE followed by autoradiography. The substrate DNA was loaded without enzyme reaction (S) or after boiling for 5 min (B) as controls.

FIGURE 4. PfuGINS is preferentially detected at the oriC region of the Pyrococcus genome during the exponential growth phase. A, the regions within 20 kb around the oriC of the Pyrococcus genome analyzed by the chromatin immunoprecipitation assay are indicated. Arrowheads indicate the ORB repeats. B, DNA binding by PfuGINS was quantified from the amount of PCR-amplified DNA by each primer set, and the results are shown as relative abundances based on the value at the 10-kb site (left side). The S.E. was obtained from three independent experiments. kb, kilobase(s).
teins related to the initiation of DNA replication in eukaryotes. Genome sequence analyses showed that the GINS complex in some archaeal organisms, including *P. furiosus*, consists of two proteins, Gins51 and Gins23, which are homologous to the eukaryotic Sld5/Psf1 and Psf2/Psf3 proteins, respectively. The two proteins form a complex with a two-to-two ratio, suggesting that the archaeal GINS is a simplified version of the GINS complex. The fact that the homolog of the GINS complex is found not only in eukaryotic cells but also in Archaea indicates that GINS is a fundamental protein that existed in the last common ancestor of Eukarya and Archaea, and therefore, investigations of the functions of the archaeal GINS will provide important information for understanding the intrinsic functions of the eukaryotic GINS. We characterized the purified GINS from *P. furiosus* and demonstrated that the helicase activity of PfuMCM is stimulated in the presence of PfuGINS in vitro. The PfuGINS also stimulates the ATPase activity of PfuMCM, and we speculate that PfuMCM changes its conformation to a suitable form for the ATPase and helicase activities by interacting with PfuGINS. It is not likely that PfuGINS helps to load the PfuMCM on the DNA substrates, because our gel retardation analyses, in which PfuMCM bound to DNA, but PfuGINS did not, showed that PfuMCM has a higher affinity for DNA than that of PfuGINS. However, we cannot exclude the possibility that PfuGINS can assist in the loading of PfuMCM onto the appropriate position of DNA by forming a complex with another unknown protein in the cells. In the case of eukaryotic DNA replication, Cdc45, at least, is required in addition to MCM and GINS for the helicase activity (8–10). However, no gene with a sequence homologous to the eukaryotic Cdc45 protein is present in the total genome sequences of many archaeal organisms in the public data base. In the case of *S. solfatarius*, GINS does not stimulate the helicase activity of SsoMCM in vitro (26). Previous reports showed that the helicase activity of SsoMCM is strong enough by itself to be detected in vitro, and SsoMCM may not need help from GINS to unwind the duplex DNA. The reason for the difference in the GINS-MCM relationships between *P. furiosus* and *S. solfatari-
cus is currently unknown. It would be interesting to elucidate the differences in the molecular mechanisms of the strand unwinding process among the archaeal organisms.

Many archaeal genomes in the data base have only the homolog of Gins51, but not that of Gins23 (Table 1). Therefore, several questions arise from this observation. Does Gins51 work as a homotetramer or is there another protein partner for the GINS function in the archaeal cells possessing only Gins51? Does Gins51 by itself interact with MCM to stimulate its helicase activity in those organisms? The helicase activity of MCM from *M. thermautotrophicus* is evident by itself, and therefore, GINS may participate in other functions besides stimulating the helicase activity of MCM in *M. thermautotrophicus* cells.

Our chromatin immunoprecipitation assay revealed that the GINS complex specifically associated with the oriC region during exponential growth. In the case of yeast DNA replication, Sld3, loaded at the origins, is required to recruit GINS to the origins (24, 31). It is not yet known whether the archaeal GINS can access oriC by itself, for example, for the specific binding to Orc1/Cdc6, or if there is some protein factor with loading activity for GINS in Archaea.

*S. solfataricus* GINS can bind to the heterodimeric primase, and it was proposed that the archaeal GINS acts as a molecular bridge between the helicase and the primase but not as a stimulating factor of these activities at the replication fork (26). On the other hand, human GINS directly interacts with and stimulates the DNA synthesis activity of the human DNA polymerase α-primase complex (Polα-primase) (32). We have not analyzed the PfuGINS-*P. furiosus* primase (p41-p46 complex (33)) interaction yet. Our preliminary analysis of direct binding by BIACORE using a Gins23-immobilized sensor chip showed no interaction with the p41-p46 complex. In the case of *S. solfataricus*, a two-hybrid analysis revealed that Gins23, but not Gins15, binds to both the small and large subunits of the primase (26). We should investigate whether Gins51 directly interacts with p41 or p46 of the *P. furiosus* primase. The gene encoding the small subunit of the primase is located immediately upstream of the gene for Gins51 in the crenarchaeal, but not the euryarchaeal, genomes, as described in a recent review article (34). It would be interesting to investigate whether the different gene organization between Crenarchaeota and Euryarchaeota has any physiological meaning.

The results of our biochemical analyses suggested that PfuGINS is probably the functional counterpart of the eukaryotic GINS complex, and it participates at least in part in some important functions for DNA replication in both initiation and fork progression in *P. furiosus*. A recent structural analysis by single-particle electron microscopy revealed the horseshoe-like shape of the human GINS complex, in which Sld5 and Psf1 formed a central core and Psf2 and Psf3 are located at each tip.
Functional Interaction of the Archaeal MCM-GINS Complex

This subunit arrangement is consistent with the previously proposed model for the yeast GINS (24) and the S. solfatarius GINS (26). The results of our gel filtration analysis support the horseshoe shape with the same subunit arrangement. However, our two-hybrid analysis revealed interactions not only with Gis51 but also with Gis23 in the GINS complex, suggesting that PfuGINS may change its conformation from a horseshoe to a ring-like structure, for example, by DNA binding, to embrace the DNA strand. Actually, rotary-shadowed electron microscopic images of the Xenopus GINS complex revealed ring-like structures (7). Furthermore, recent structural studies showed that the crystal structures of the human GINS complex resemble a trapezoid (36) or elliptical shape (37, 38), in which the four subunits interact with each other.

At this stage we cannot conclude that the MCM and GINS complexes move together with the replication fork after the initiation step in P. furiosus cells, because we have not yet isolated the stable MCM-GINS complex. Therefore, we propose two models in which GINS forms the core complex with MCM and other unknown proteins for the active replicative helicase (Fig. 9A) or GINS leaves the MCM complex just after changing the conformation of the MCM to activate its helicase activity (Fig. 9B) in P. furiosus. An in vitro replication system, which we have been trying to reconstitute using a P. furiosus oriC plasmid and replication-related proteins, will be the most powerful tool to elucidate the concrete functions of the GINS complex during the initiation and elongation processes of DNA replication.

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