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ORIGINAL PAPER

The GINS complex from the thermophilic archaeon, *Thermoplasma acidophilum* may function as a homotetramer in DNA replication

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Abstract The eukaryotic GINS heterotetramer, consisting of Sld5, Psf1, Psf2, and Psf3, participates in "CMG complex" formation with mini-chromosome maintenance (MCM) and Cdc45 as a key component of a replicative helicase. There are only two homologs of the GINS proteins in Archaea, and these proteins, Gins51 and Gins23, form a heterotetrameric GINS with a 2:2 molar ratio. The *Pyrococcus furiosus* GINS stimulates the ATPase and helicase activities of its cognate MCM, whereas the *Sulfolobus solfataricus* GINS does not affect those activities of its cognate MCM, although the proteins bind each other. Intriguingly, *Thermoplasma acidophilum,* as well as many euryarchaea, have only one gene encoding the sequence homologous to that of archaeal Gins protein (Gins51) on the genome. In this study, we investigated the biochemical properties of the gene product (TaGins51). A gel filtration and electron microscopy revealed that TaGins51 forms a homotetramer. A physical interaction between TaGins51 and TaMcm was detected by a surface plasmon resonance analysis. Unexpectedly, TaGins51 inhibited the ATPase activity, but did not affect the helicase activity of its cognate MCM. These results suggest that another factor is required to form a stable helicase complex with MCM and GINS at the replication fork in *T. acidophilum* cells.

Keywords Archaea · DNA replication · Replisome · DNA helicase · Protein interaction · Thermoacidophile

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Introduction

The molecular mechanisms of DNA replication have been actively studied in a variety of organisms from the three domains: Bacteria, Eukarya, and Archaea (reviewed in Barry and Bell 2006; Masai et al. 2010; Mott and Berger 2007). No sequence conservation exists among the proteins involved in DNA replication between Bacteria and Archaea/Eukarya, and the two DNA replication apparatuses are considered to have evolved independently (Leipe et al. 1999). Based on these observations, the archaeal DNA replication machinery is most likely a simplified version of the eukaryotic system. Therefore, studies of

archaeal DNA replication should help to elucidate the mechanisms common to both the archaeal and eukaryotic systems, in addition to clarifying their intrinsic importance in the evolution of the DNA replication apparatus.

DNA replication begins with the activation of the replication origin. In the eukaryotic replication system, minichromosome maintenance (MCM), forming a hexameric complex (Mcm2-7), is considered to be the core of the replicative helicase. MCM is recruited to the origin to assemble the pre-replication complex (pre-RC) during the G1 phase of the cell cycle, and starts to unwind the DNA bidirectionally from the origin, to allow the replication forks to progress during the S phase.

The GINS complex was originally identified in *Saccharomyces cerevisiae* as essential protein factors for the initiation of DNA replication (Takayama et al. 2003). The complex consists of four different proteins, Sld5, Psf1, Psf2, and Psf3, and was named after the Japanese numbers go-ichi-ni-san, meaning 5-1-2-3 of each subunit. Immunoprecipitation experiments using *Xenopus* oocyte extracts detected a physical interaction between MCM, Cdc45, and the GINS complex proteins (Kubota et al. 2003). Subsequently, the replisome progression complex (RPC) including these three components was isolated from yeast (Gambus et al. 2006), *Drosophila* (Moyer et al. 2006), and *Xenopus* oocyte extracts (Pacek et al. 2006). The complex of Cdc45, MCM, and GINS is referred to as the "CMG complex" after their initials (Moyer et al. 2006), or the "unwindosome" (Pacek et al. 2006). The immunoaffinity-purified CMG complex displayed ATP-dependent unwinding activity of a 40 nt oligonucleotide annealed to single-stranded DNA (Moyer et al. 2006).

In contrast to the heterohexameric eukaryotic MCM complex, most archaeal organisms contain only one gene encoding an MCM homolog (Barry and Bell 2006). MCM complexes in various forms, including hexamer, heptamer, double-hexamer, and a filamentous form, from the single subunit Mcm proteins from four archaeal organisms, *Methanothermobacter thermautotrophicus* (Chen et al. 2005; Chong et al. 2000; Costa et al. 2006a, b; Gambus et al. 2006; Gomez-Llorente et al. 2005; Kelman et al. 1999; Shechter et al. 2000; Yu et al. 2002), *Sulfolobus solfataricus* (Brewster et al. 2008; Carpentieri et al. 2002; McGeoch et al. 2005), *Archaeoglobus fulgidus* (Grainge et al. 2003), and *Thermoplasma acidophilum* (Haugland et al. 2006, 2008b; 2009) have been characterized to date. The archaeal MCMs have a distinct DNA helicase activity by themselves in vitro, and therefore, these MCMs may function as the replicative helicase in archaeal cells. Current understanding of the structure–function relationships of the archaeal MCM helicase is summarized in the review article (Sakakibara et al. 2009). Recently, we reported that *P. furiosus* MCM is recruited onto the *oriC* region in an Orc1/Cdc6-dependent manner based on the results of an in vitro recruitment assay (Akita et al. 2010).

The amino acid sequences of the four subunits in the GINS complex share some conservation, suggesting that they are ancestral paralogs (Makarova et al. 2005). The ORFs encoding the homologs of eukaryotic GINS proteins were predicted in the archaeal genomes by finely detailed bioinformatic analyses, although most archaea encode a single gene of this family (Makarova et al. 2005). A predicted protein was detected in the crenarchaeon *S. solfataricus,* by a yeast two-hybrid screening for interaction partners of MCM, and was designated as Gins23 (Marinsek et al. 2006). Furthermore, another subunit was identified by the mass spectrometry analysis of an immunoaffinity-purified native GINS complex from a *S. solfataricus* cell extract, using an anti-Gins23 antibody, and was designated as Gins15, based on its closer similarity to Sld5 and Psf1. From these studies, the *S. solfataricus* GINS complex, a tetrameric structure with a 2:2 molar ratio of Gins23 and Gins15, was identified (Marinsek et al. 2006). Our further search of archaeal genomes, based on the sequences of the two Gins proteins from *S. solfataricus* revealed that crenarchaeal organisms have two homologs and most euryarchaeal organisms, except Thermococcales, have only one homolog (Yoshimochi et al. 2008). We characterized the complex of the Gins homologs from *Pyrococcus furiosus*, which includes Gins23 and Gins51 at a 2:2 ratio as the first report describing the euryarchaeal GINS (Yoshimochi et al. 2008). Our chromatin immunoprecipitation assays revealed that *P. furiosus* GINS (PfuGINS) is detected preferentially at the *oriC* region of *P. furiosus* chromosomal DNA during the exponential growth phase, as also reported for *P. furiosus* MCM (PfuMCM) (Matsunaga et al. 2007; Yoshimochi et al. 2008). It is especially interesting that the DNA helicase activity of PfuMCM is clearly stimulated by the addition of the PfuGINS complex (Yoshimochi et al. 2008). We named the GINS subunit proteins Gins23 and Gins51 (instead of Gins15), consistent with the original nomenclature of GINS from 5-1-2-3.

The molecular evolution of the GINS complex and its concrete functions in the initiation and elongation processes of archaeal and eukaryotic DNA replication are very interesting subjects. We focused on the Gins protein that is found as the sole homolog in the whole genome of euryarchaeal organisms, and thus analyzed the biochemical properties of the Gins protein from *Thermoplasma acidophilum* as an example in this study. Extensive characterizations of the two Orc1/Cdc6 proteins and the Mcm protein derived from *T. acidophilum* were previously reported (Haugland et al. 2006, 2008a, b, 2009). Gins is the third DNA replication initiation-related protein from *T. acidophilum* that has been characterized biochemically.

Materials and methods

Cultivation of *T. acidophilum* cells

T. acidophilum was cultivated in 2 l of medium, containing yeast extract, casamino acids, $(NH_4)_2SO_4$, NaCl, KH_2PO_4, $MgSO_4$, and $CaCl_2$ (pH was adjusted to 1.8 by H_2SO_4), at 56°C with shaking as described previously (Yasuda et al. 1995). The total cell extract of *T. acidophilum* was prepared by sonication of the cells in a buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol for 10 min (5 s on-5 s off).

Cloning of the gene encoding the Gins51 homolog

The gene encoding *gins51*, corresponding to Ta1042 in the *T. acidophilum* genome database, was amplified by polymerase chain reaction (PCR) directly from *T. acidophilum* genomic DNA, prepared by a standard phenol–chloroform extraction, using two oligonucleotides, 5′-dAAACATA TGAAGCCGGCAGATATCGATAAG-3′ and 5′-dAAA GCGGCCGCTCATTGATCCAGAACCAGCGCCAC-3′, as the forward and reverse primers, respectively. The 50 µl reaction mixture contained 20 mM Tris–HCl, pH 8.0, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), 2.5 mM each dNTPs, 50 ng of genomic DNA, 0.4 µM each of forward and reverse primers, and 2.5 U cloned Pfu DNA Polymerase (Stratagene). The PCR was performed with 30 cycles at 57°C for 30 s, and 72°C for 70 s, after denaturation at 98°C for 10 s. The amplified gene was further incubated with *Taq* DNA Polymerase (*TaKaRa Taq,* TAKARA BIO) to add adenosine to its 3′-termini, and then cloned into the pGEM-T Easy vector (Promega). The forward and reverse primers described above have the recognition sequences for *Nde*I and *Not*I, respectively, and therefore, the cloned gene was digested by *Nde*I–*Not*I and inserted into the corresponding sites of pET21a (Novagen). The plasmid thus generated was designated as pET21a-TaGisn51.

Overproduction and purification of TaGins51 and TaMcm

To obtain the highly purified TaGins51 at a high concentration (>10 mg/ml), BL21 CodonPlus (DE3)-RIL (Stratagene) cells carrying pET21a-TaGins51 were cultivated in 4 l of LB medium containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37°C. When the culture reached an $A_{600} = 0.50$, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the expression of the *gins51* gene and cultivation was then continued overnight at 25°C. The cells were harvested by centrifugation (10 min, 5,180×*g*), and were disrupted by sonication for 10 min in buffer A (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, and 10% glycerol). The soluble cell extract obtained by centrifugation (10 min, 23,708×*g*) was heated at 60°C for 20 min. The heat-resistant fraction, obtained by centrifugation (10 min, 23,708×*g*), was treated with 0.5% polyethyleneimine, and the insoluble fractions, obtained by centrifugation (10 min, 23,708×*g*), were resuspended in buffer A containing 0.5 M $(NH_4)_2SO_4$, and subjected to chromatography on a 5 ml HiTrap Phenyl HP column (GE Healthcare), which was developed with a 0.5–0 M $(NH_4)_2SO_4$ linear gradient. The protein fractions, eluted at 0.13 M $(NH_4)_2SO_4$, were diluted with buffer A containing 1.0 M $(NH_4)_2SO_4$ to adjust the concentration of $(NH_4)_2SO_4$ to 0.5 M and were loaded again onto a 5 ml HiTrap Phenyl HP column. TaGins51-containing fractions were dialyzed against buffer A, and then were loaded onto a 5 ml HiTrap Q HP column (GE Healthcare), which was developed with a 0–0.5 M linear gradient of NaCl. To concentrate the purified TaGins51, the positive fractions, which eluted at 0.3 M NaCl, were diluted with buffer A and loaded onto a 1 ml HiTrap Q HP column (GE Healthcare). The protein fractions that eluted at 0.4 M NaCl, were pooled and used in this study. The TaMcm protein was prepared using an expression plasmid as described previously (Haugland et al. 2006). The protein concentrations of TaGins51 and TaMcm were determined using absorbances at 280 nm and extinction coefficients of 11,920 M^{-1} cm^{-1} for TaGins51 as a monomer and 49,530 M^{-1} cm^{-1} for TaMcm as a monomer, respectively.

Western blot analysis

The protein samples were separated by 12% SDS-PAGE. The proteins on the gel were electroblotted onto a polyvinylidene difluoride membrane (BioRad), and reacted with the anti-TaGins and anti-TaMcm antisera (prepared by injecting purified recombinant proteins into the rabbits). The proteins were visualized by an enhanced chemiluminescence system (Millipore) and quantified with an LAS-3000 mini image analyzer (FUJIFILM).

Gel filtration and glycerol gradient centrifugation

Gel filtration chromatography was performed using the SMART system (GE Healthcare). The purified recombinant TaGins51 (300 pmol) was applied to a Superdex 200 3.2/30 column (GE Healthcare), pre-equilibrated with buffer A containing 0.15 M NaCl and the chromatography was developed using the SMART system. The molecular masses of the proteins were estimated

from the elution profiles of standard marker proteins (BioRad), including thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), and myoglobin (17,000). TaGins51 was also subjected to glycerol gradient centrifugation. The purified TaGins51 protein (50 μg) was sedimented through a 5–20% (w/v) glycerol gradient at 43,000 rpm at 4°C for 24 h in a Beckman SW60 Ti rotor. The standard marker proteins, including bovine serum albumin (67,000), ovalbumin (43,000), Chymotrypsinogen A (25,000), and Ribonuclease A (13,700) from GE Healthcare, were sedimented under identical conditions.

Electron microscopy and single particle image analysis of TaGINS

The purified TaGINS complex was diluted to a final protein concentration of 5 μg/ml, in a buffer containing 50 mM Tris–HCl, pH 8.0 and 40 mM NaCl. A 3 μl aliquot of the sample solution was applied to a copper grid supporting a continuous thin-carbon film, left for 1 min, and then stained with three drops of 2% uranyl acetate. The negatively stained complex was examined with a Tecnai T20 electron microscope (FEI), operated at an accelerating voltage of 200 kV. Images were recorded by an Eagle 2 K CCD camera (FEI), with a pixel size of 2.76 Å/pixel. A low dose system was used to reduce the electron radiation damage of the sample. A total of 982 images of the TaGINS complex were selected, using the BOXER program in EMAN (Ludtke et al. 1999). The two-dimensional class averages were obtained using the refine tool of EMAN, assuming 6 classes.

ATPase assay

The purified TaMcm (1 pmol as the hexamer) was incubated in a 15 μl of reaction mixture containing 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 40 μM ATP, 0.33 pmol of [γ-^{32}P]ATP and increasing amounts of TaGins51 (0.5, 1, 2, and 4 pmols as the tetramer) in the presence or absence of 5 μM nucleotides (base pairs) M13 single-stranded (ss) or double-stranded (ds) DNA. After incubation at 59°C for 15, 30, 45, and 60 min, an aliquot (1 μl) was spotted onto a polyethyleneimine-cellulose thin layer chromatography plate to separate the ATP and the released Pi using a solvent containing 1 M formic acid and 0.5 M LiCl. The ATPase activity was quantified by an image analyzer (FLA5000, Fuji Film). The amount of Pi in the reaction without the enzyme was deducted from that of each reaction as the spontaneous hydrolysis at the high temperature. The standard deviation was calculated from three independent experiments.

Helicase assay

The helicase activity of TaMCM was measured in 15 μl reaction mixtures containing 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, 3.3 mM ATP, 50 fmol of the ^{32}P-labeled pseudo-Y DNA substrate, composed of 5′-^{32}P-labeled F3-d47 (5′-dAGCTATGAC CATGATTACGAATTGCTTGGAATCCTGACGAACTG TAG-3′) and 49N (5′-dAGCTACCATGCCTGCACGA ATTAAGCAATTCGTAATCATGGTCATAGCT-3′) as described previously (Komori et al. 2004, Fujikane et al. 2006), and 1 pmol of TaMcm (as the hexamer) with increasing amounts of TaGins51 (0.5, 1, 2, and 4 pmol, as the tetramer). After an incubation at 59°C for 10 min, the samples were immediately transferred to ice and 5 μl of 4× stop buffer (100 mM EDTA, 1% SDS, 50% glycerol, 0.1% bromophenol blue, and 0.1% xylene cyanol) was added. An aliquot (7 μl) was loaded onto a 10% polyacrylamide gel in 0.5× TBE (45 mM Tris–borate, 1 mM EDTA) and electrophoresed at 15 mA for 40 min. The gel image was visualized by autoradiography and the helicase activity was quantified by an image analyzer, Typhoon Trio+ (GE Healthcare).

Immunoprecipitation assays

A 20 μl portion of rProtein A Sepharose (GE Healthcare Biosciences) was washed thrice with PBS-T (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 0.1% Tween 20), mixed with PBS-T containing 10 μl of each antiserum, and incubated at room temperature for 1 h on a rotary shaker. Each mixture was washed twice with PBS-T, and then twice with 0.2 M triethanolamine, pH 8.0. The antibody was cross-linked to the rProtein A Sepharose with dimethyl suberimidate 2 HCl (DMS, PIERCE), according to the manufacturer's protocol. Pre-immune antiserum was used as negative control experiments. After equilibration of the antibody-conjugated rProtein A Sepharose with PBS-T, a 400 μl aliquot of *T. acidophilum* cell extract (70 mg/ml) was added, and the mixture was incubated for 30 min on a rotary shaker. The precipitates were washed thrice with PBS-T, and the immunoprecipitated proteins were eluted with 40 μl of gel loading solution (50 mM Tris–HCl, pH 6.8, 10% glycerol, 1% β-mercaptoethanol, 0.2 mg/ml bromophenol blue, 2% SDS). Three microliters of the elution solutions were subjected to the western blot analysis.

Gel-retardation assay

The oligonucleotide 49N (the sequence was described above) was labeled with ^{32}P at the 5′-terminus and used as the ssDNA probe. The reaction mixtures (20 μl), containing 20 mM HEPES–NaOH, pH 7.5, 10 mM $MgCl_2$,

0.1 mg/ml BSA, 50 fmol of 5′-^{32}P-labeled DNA substrate, and proteins (TaMCM and TaGins51) in the presence of 1 mM ATPγS, were incubated at 59°C for 10 min. Glutaraldehyde was then added to a final concentration of 0.02% and the reactions were incubated at room temperature for 10 min to crosslink the DNA substrate and the DNA-bound proteins. For the antibody supershift, the reaction mixtures were further incubated with either anti-TaMcm or anti-TaGins51 antiserum at room temperature for 10 min. Loading buffer (5×), containing 20 mM HEPES–NaOH, pH 7.5, 50% glycerol, 0.1% bromophenol blue, and 0.1% xylene cyanol, was added to the reaction mixtures and an aliquot (7 µl) was fractionated on a 4% polyacrylamide gel in 1× TAE (40 mM Tris–acetate, 1 mM EDTA) by electrophoresis at 15 mA for 40 min, and then visualized by an imager (Typhoon Trio+, GE Healthcare).

Surface plasmon resonance analysis

The surface plasmon resonance (SPR) analysis was performed using BIACORE J system (GE Healthcare). To detect the direct interaction between TaGins51 and TaMcm, His-tagged TaMcm was immobilized on a Sensor Chip NTA according to the manufacturer's protocol (GE Healthcare), and then TaGins51 was loaded as the analyte onto the chip. The BIACORE analysis was conducted at 25°C in 10 mM HEPES–NaOH, pH 7.4, containing 50 µM EDTA, and 0.05% Tween 20 at a flow rate of 15 µl/min. The immobilization of TaMcm resulted in 3,000 RU. The purified TaGins51 protein was loaded onto the chip with the same running buffer as described above at a flow rate of 30 µl/min. To measure the kinetic parameters, various concentrations of TaGins51 (0.5, 1.25, 3.13, 6.25, and 12.5 µM as the tetramer) were loaded, and the apparent equilibrium constant (K_D) for TaMcm-TaGins51 was calculated from the association and dissociation curves of the sensorgrams, using the BIAevaluation program (GE Healthcare).

Results

The *T. acidophilum* genome encodes only one homolog of the archaeal Gins protein

A comprehensive search for Gins protein homologs in archaeal genomes revealed that only *Thermococcal* organisms in Euryarchaeota, in contrast to all of the crenarchaeal genomes, except for *Thermofilum pendes*, have two genes encoding Gins homologs, as we reported previously (Yoshimochi et al. 2008) and as also discussed in a recent review article (MacNeill 2010). A gene encoding a sequence with some similarity to the archaeal Gins51 protein is present in the *T. acidophilum* genome. The Ta1042 gene is not part of an operon, and is not linked with the gene for the small subunit of the primase as usually observed in the archaeal genomes. To show the diversity of the Gins proteins in Archaea, we aligned the amino acid sequences of the Gins proteins from representative archaea. It was obvious that the sequence conservation is very weak among the archaeal Gins proteins. One notable characteristic is the distinctly long linker region between Domains A and B in the proteins from the archaea that have only one Gins homolog (Fig. S1), as pointed out for the Gins51 proteins from haloarchaeal organisms (MacNeill 2009). Based on these observations, it is interesting to investigate whether the sole homolog functions as GINS in the archaeal cells for a basic understanding of the GINS complex in terms of its evolution and functions.

Purification of the TaGins51 and TaMcm

We cloned the gene corresponding to Ta1042 and expressed it in *E. coli* cells. The overexpressed product, designated TaGins51, was detected by an SDS-PAGE analysis of the whole cell extract. TaGins51 was further detected in the supernatant fraction after heat treatment of the total cell extract, and was then purified to homogeneity by subsequent polyethyleneimine treatment (to remove nucleic acids) followed by two types of column chromatography, as described in the "Materials and methods". A Coomassie Brilliant Blue stained gel of the purified TaGins51 protein is shown in Fig. 1. The band appeared as a much larger protein than that predicted from the calculated molecular weight of Ta1042 (MW: 26,994.5) as compared with the size marker proteins, probably because of some structural feature. Starting from a 1 l culture, 13.1 mg of homogeneous TaGins51 was obtained. The Mcm protein from *T. acidophilum* was previously biochemically characterized as a 3′–5′ helicase with DNA-dependent ATPase activity (Haugland et al. 2006, 2008b, 2009). To investigate physical and functional interactions with TaGins51, we purified TaMcm as a recombinant protein according to the published procedure. TaMcm was also produced efficiently in *E. coli* cells and the protein was purified to homogeneity (Fig. 1b) with a good yield (2 mg/l culture).

Estimation of the amount of TaGins51 in *T. acidophilum* cells

To estimate the amount of Gins51 protein produced in growing *T. acidophilum* cells, the band intensity corresponding to TaGins51 in the cell extract was quantified by a western blot analysis. The amount of TaGins51 in the cells was calculated using a standard curve obtained from

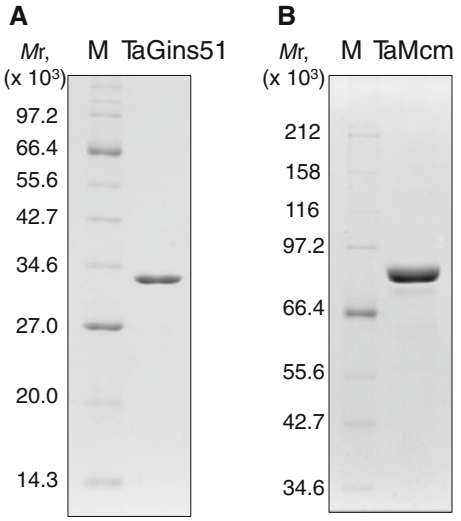

Fig. 1 Purification of TaGins51 and TaMcm proteins. The purified TaGins51 (**a**) and TaMcm (**b**) proteins were subjected to 12.5 and 7.5% SDS-PAGE, respectively, and were stained by Coomassie Brilliant Blue. *M* molecular mass standards (New England Biolabs Inc.)

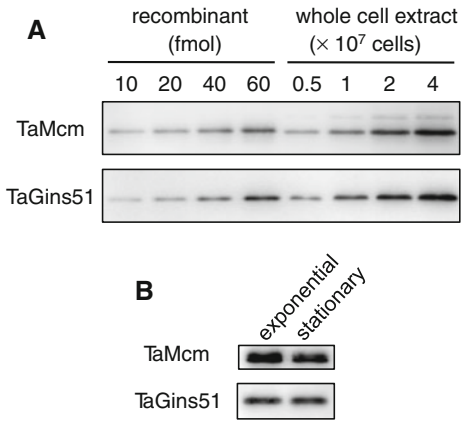

Fig. 2 Estimation of the amount of TaGins51 in *T. acidophilum* cells. **a** *T. acidophilum* cells at exponential and stationary phases were harvested, and the cell extracts from 0.5 to 4×10^7 cells were subjected to 12% SDS-PAGE, followed by western blot analyses using anti-TaMcm (*upper panel*) and anti-TaGins51 (*lower panel*) antisera on the left side. The amounts of TaGins51 and TaMcm in *T. acidophilum* cells were calculated from standard curves representing known quantities (10–60 fmol) of each purified recombinant protein as shown in the *left side*. **b** Comparison of the amounts of TaGins51 and TaMcm in the cells from the exponentially growing and stationary phases

the band intensities of serial dilutions of purified TaGins51 (Fig. 2a), and was estimated to be about 2,000 molecules per cell. The amount of TaMcm was also quantified by the same type of experiment that was performed in parallel. The number of TaMcm molecule per cell was also about 2,000, which is consistent with the previously reported value (1,000–1,800) (Haugland et al. 2009). The faint bands reacting with anti-TaMcm antibody are observed above the band of TaMcm in the lanes containing the whole cell extracts might be the phosphorylated form as reported for the Mcm protein from *Aeropyrum pernix* (Atanassova and Grainge 2008). We also compared the amounts of TaGins51 and TaMcm between exponential and stationary phases of *T. acidophilum* cell cultures. As shown in Fig. 2b, no difference was observed between the two growth phases for either protein. The amount of Mcm in *P. abyssi* is also the same between the two phases (Matsunaga et al. 2001).

TaGins51 forms a homotetrameric complex

One of the most interesting issues in the characterization of the TaGins51 protein is its status in solution. To address this question, we performed gel filtration chromatography. The purified TaGins51 protein was applied to a Superdex 200 column and a single peak with symmetrical shape was obtained as shown in Fig. 3a. The estimated molecular weight of the protein from the elution position is 118,000, which corresponded to the size of a tetrameric complex of the TaGins51 protein monomer, 26,995. A glycerol gradient separation experiment was performed in parallel using the same protein sample. However, the TaGins51 protein sedimented to the position corresponding to the molecular weight of the protein monomer (Fig. 3b). The linear calibration curves ($r^2 > 0.98$) were obtained based on four calibration proteins from both the gel filtration and the glycerol gradient sedimentation experiments. Furthermore, these results were reproducible from three independent experiments. To confirm that TaGins51 forms a tetramer in solution, the protein sample was subjected to electron microscopy (EM). Well-dispersed particles with the same size were predominant in the EM image. As shown in Fig. 4, the size of the class averaged images from our EM (b) is the same size as that of the crystal structure of the heterotetrameric human GINS with a rhombus-like shape of 70 Å square (c), when the structure was observed from a certain direction. These results indicate that TaGINS is the homotetramer of TaGins51 in solution, but the complex is not strong enough to form a stable complex to persist under a centrifugal force of 190,000×*g* (average).

Physical interaction between TaGINS and TaMCM

One of the roles of the GINS complex in eukaryotic DNA replication process is to function as a member of the helicase complex for replication fork progression. Therefore, we investigated the direct interaction between TaGINS and TaMCM in vitro by a SPR analysis. As shown in Fig. 5a, the resonance units (RU) increased when purified TaMcm was immobilized on the Biacore NTA sensor

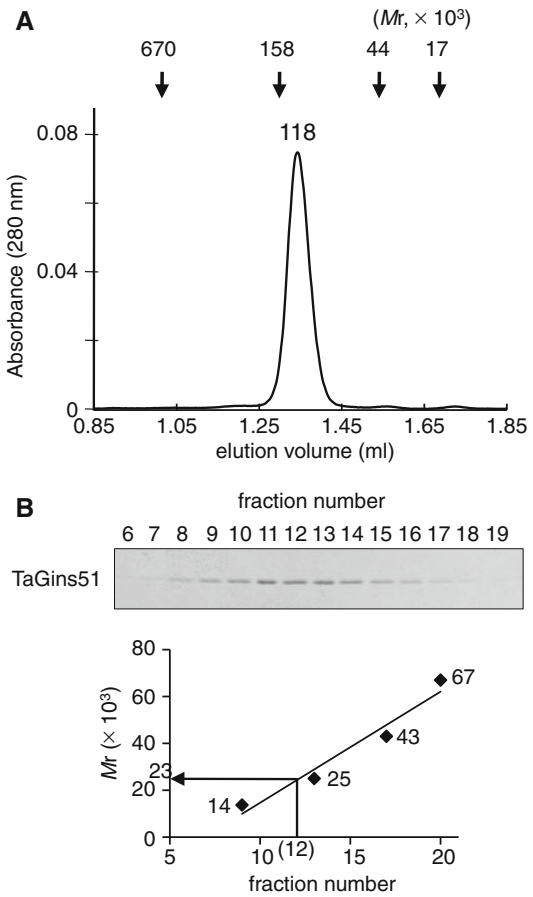

Fig. 3 Oligomeric state of TaGins51 in solution. **a** Gel filtration analyses of purified TaGins51. The protein solution was fractionated on a Superdex-200 column. The elution profiles monitored by the absorbance at 280 nm are shown. The oligomeric states of the TaGINS complex were predicted by calculating the molecular weights from the retention volume in the chromatography. **b** Glycerol gradient centrifugation analysis. Purified TaGins51 was sedimented for 24 h at 4°C through a 5–20% glycerol gradient. The standard curve was obtained with marker proteins on a parallel gradient. The molecular sizes of each marker protein ($\times 10^3$) are shown with the plots

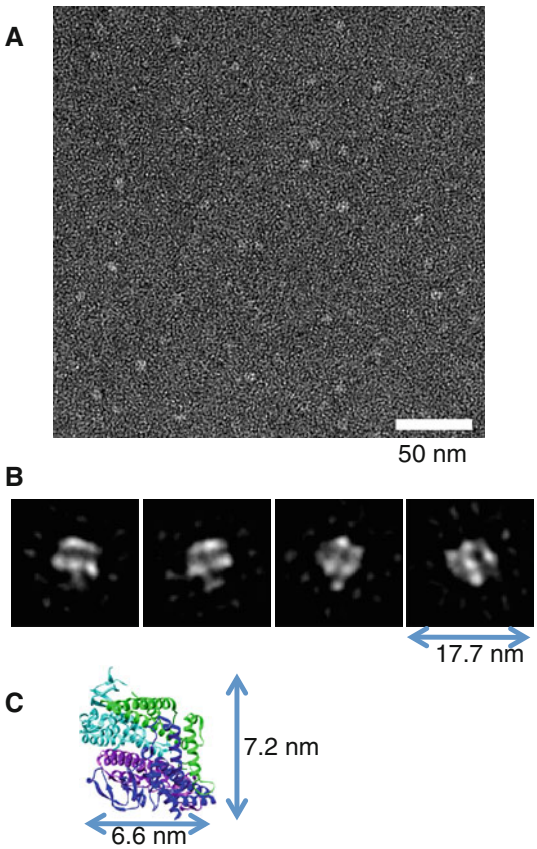

Fig. 4 Electron microscopy of TaGINS. **a** Electron micrograph of the negatively stained TaGINS complex (*scale bar* 50 nm). **b** Class averaged images of TaGINS complex. The side length of the individual images is 17.7 nm. **c** Crystal structure of human GINS complex (tetramer) from Kamada et al. 2007

chip and increasing concentrations of TaGins51 were passed over the sensor chip. The calculated K_D from this experiment was 3.24×10^{-5} M, indicating that TaGins51 directly binds TaMcm, but the affinity is not strong enough for a stable complex. To confirm that TaGINS-TaMCM forms a complex in cells, an immunoprecipitation experiment was performed using a *T. acidophilum* cell extract and antibodies against TaGins51 and TaMcm. As shown in Fig. 5b, the TaMcm band was detected in the fraction precipitated with the anti-TaGins51 antibody. Conversely, TaGins51 was coprecipitated with TaMcm by an anti-TaMcm antibody. To investigate whether TaGINS and TaMCM form a more stable complex in the presence of DNA, we performed an in vitro gel-retardation assay using ^{32}P-labeled DNA as a probe. As shown in Fig. 5c, the DNA band was clearly shifted in the presence of TaMcm, but not TaGins51, indicating that TaGINS has little affinity for DNA by itself, as suggested previously for PfuGINS (Yoshimochi et al. 2008). The shifted band was further shifted with anti-TaMcm, but not with anti-TaGins51 nor pre-immune antiserum (data not shown). When increasing amounts of TaGins51 were added to the reaction solution containing a constant amount of TaMcm, the band affected by TaMcm-binding shifted further. This shifted band was further shifted with either anti-TaMcm or TaGins51, but not with pre-immune antiserum (data not shown). These results indicate that TaGINS can bind to TaMCM on the DNA strand. However, these bandshifts mediated by protein-binding strictly required glutaraldehyde, a cross-linking reagent, suggesting that the TaMCM-DNA and TaMCM–TaGINS-DNA complexes are inherently unstable and dissociate easily.

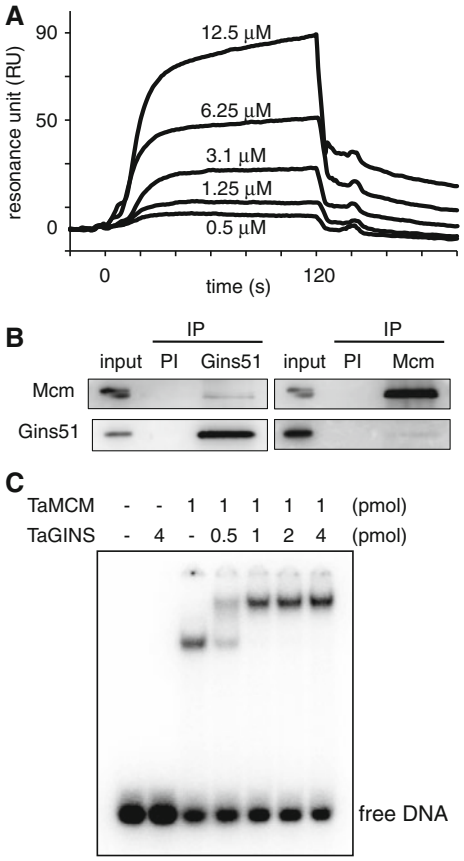

Fig. 5 Physical interaction between TaGins51 and TaMcm. **a** An SPR analysis was performed using a BIACORE system to analyze the TaGins51–TaMcm interaction. Purified TaMcm was immobilized on a sensor chip, and various concentrations of TaGins51 were injected for 120 s. **b** Immunoprecipitation analyses were performed to confirm the formation of a complex including MCM and GINS in the *T. acidophilum* cell extract. The immunocomplexes were captured with anti-TaMcm and anti-TaGins51 antisera, respectively, from the whole cell extract (as shown on the *top*), and were subjected to SDS-PAGE, followed by western blot analyses using these antisera (shown on the *left side*). The whole cell extracts without immunoprecipitation (*input*) or precipitated with the rProtein A Sepharose treated with pre-immune antiserum (*PI*) were also loaded as positive and negative controls, respectively. **c** DNA binding activity of TaMcm and TaGins51. The TaMcm and TaGins51 proteins were incubated with a 49 nt ^{32}P-labeled single-stranded DNA at 59°C for 10 min followed by crosslinking with glutaraldehyde, and the reaction products were analyzed by 4% PAGE. Increasing amounts of TaGins51 were added to the constant amount of TaMcm as shown at the *top* of each *lane*

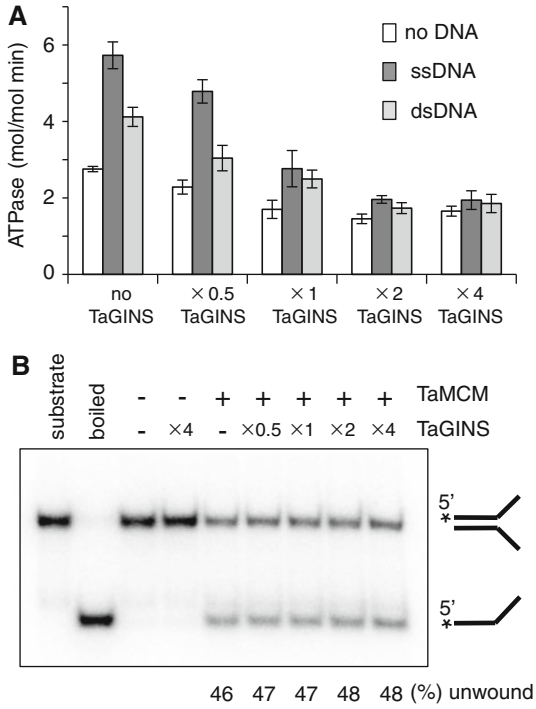

Fig. 6 Effect of TaGins51 on the ATPase and helicase activities of TaMcm. Purified TaGins51 was added in increasing amounts to the assay mixtures for the ATPase and the helicase activities of TaMCM, as described in the "Materials and methods". **a** The ATPase activity is indicated as the amount of released Pi by the constant amount of the TaMcm protein (1 mol) for 60 min incubation in each reaction condition in the absence or presence of ss or ds DNA. The products were confirmed to increase proportionally until 60 min. The assays were carried out thrice independently. **b** The helicase activity is indicated at the *bottom* of the panel as the relative amount of unwound DNA (%) in each reaction condition with or without TaGINS. The DNA substrates, before and after boiling, were loaded in parallel as a negative and a positive control of the unwinding reaction

TaGINS inhibits the ATPase activity, but not the helicase activity, of TaMCM

The recombinant TaMCM protein has an ATP-dependent helicase activity (Haugland et al. 2006, 2008b, 2009). To understand the physiological meaning of the physical interaction between TaGINS and TaMCM, we investigated the effect of TaGINS on the ATPase and helicase activities of TaMCM in vitro. As reported previously, the ATPase activity of TaMCM was stimulated by about 2-fold in the presence of single-stranded DNA (Fig. 6). Unexpectedly, this DNA-dependent ATPase activity became weaker with increasing amount of the TaGins51 protein. This is in contrast to the case of *P. furiosus*, in which PfuGINS stimulated the ATPase of PfuMCM (Yoshimochi et al. 2008). To examine whether this decreased ATPase activity affects the helicase activity of TaMCM, an in vitro DNA unwinding assay was performed. Surprisingly, the helicase activity of TaMCM was the same in the presence and absence of TaGINS in the reaction solution. The results from the ATPase and helicase activities suggest that TaGINS changes the conformation of TaMCM by direct interaction, but the two complexes then dissociate quickly to restore the MCM to its own conformation.

Discussion

We cloned the gene corresponding to the homolog of the archaeal Gins51 protein from *T. acidophilum* and characterized the gene product in vitro. The aim of this work was to determine whether the single homolog works as the GINS complex. Our experimental result showed that TaGins51 forms a homotetramer and interacts with TaMCM. This is the first example of a homotetrameric GINS complex. The archaeal complexes contain fewer kinds of subunit proteins as compared with the eukaryotic complexes, as commonly seen for MCM and Replication factor C (RFC). In the case of the pentameric RFC complex, most archaeal organisms have only two subunit proteins, RFCL and RFCS, forming a complex with 1:4 ratio, in contrast to five different proteins, RFC1-5, in the eukaryotic RFC complex. We recently characterized an RFC with three subunits, RFCS1, RFCS2, and RFCL forming a 3:1:1 complex from *Methanosarcina acetivorans,* a methanogenic archaeon, and proposed an evolutionary interest of this molecule (Chen et al. 2009). Comparative analyses of the structure and functions of the complex with a different number of subunit proteins are helpful for understanding of the necessity and diversity of the complex in detail.

The tetrameric form is widely conserved among the GINS complexes from Eukarya and Archaea, and the homotetrameric form is valuable as the simplest structure for comparative studies of the GINS molecule from an evolutionary viewpoint. However, critical questions regarding homotetrameric structure of the GINS complex remain. How is the oligomerization regulated to form only the tetramer? If the multiple functions of GINS depend on the properties of the individual subunit proteins, then how does the homotetrameric GINS accomplish these functions with a single subunit? Our gel filtration profile revealed that TaGins51 was eluted as a single peak with perfect symmetrical shape, indicating that its predominant oligomeric state is the tetramer. However, a glycerol gradient centrifugation experiment showed that TaGins51 broadly sedimented and was mainly detected at the monomer position. We previously reported that PfuGINS was obtained as a tetramer, from both gel filtration and glycerol gradient centrifugation under the same conditions as in this study (Yoshimochi et al. 2008). We also detected the tetramer form of GINS (Gins23-Gins51 with 2:2 ratio) from *Thermococcus kodakaraensis* by glycerol gradient centrifugation (Ishino et al. unpublished). These results indicate that the homotetrameric complex of TaGINS is clearly less stable than the GINS heterotetramer with two different subunits. Further structural analyses including crystallographic studies are required to understand the interaction mode of each subunit in the homotetrameric structure of TaGINS. The EM image showed that the size of the TaGINS molecule is similar to that of the heterotetramer form of the human GINS crystal structure, suggesting that the three-dimensional structure of GINS is basically conserved between Archaea and Eukarya. Some mechanistic regulation should exist in the structure of the homotetrameric GINS, such as TaGINS, to prevent polymerization larger than tetramer.

In terms of the functional difference between the archaeal and eukaryotic GINS proteins, some important roles distinct from DNA replication in the S-phase of the cell cycle have been reported for the eukaryotic GINS. These functions are related to mitosis and cytokinesis (Huang et al. 2005), and GINS is also required for the progression of a number of developmental processes in vertebrates (Obama et al. 2005; Ueno et al. 2005, 2009; Walter and Henry 2004; Walter et al. 2008). These functions may have evolved in relation to heterocomplex formation by GINS, to function in more complicated biological processes. For the situation of the archaeal GINS structure, a reasonable scenario has been proposed for the evolution of the *gins* gene in relation to the adjacent genes for Primase and Mcm in Archaea. In this scenario, the genes encoding both Gins51 and Gins23 existed in the last common archaeal ancestor, and the gene encoding Gins23 was lost from many lineages during subsequent archaeal evolution (Swiatek and Macneill 2010). The discrimination between Gins51 and Gins23 may not be critical to maintain the viability of Archaea. However, it is possible that a Gins23-like protein with a highly diverse sequence exists, and that Gins51 forms a heterocomplex with it in the archaeal cells that only have the Gins51 homolog. We are trying to purify TaGins51 from *T. acidophilum* cells by immunoaffinity chromatography using an anti-TaGins51 antibody to isolate the native TaGins51-containing complexes. These experiments will reveal the genuine shape of the GINS protein.

The results of this study should be interpreted based on the functional GINS complex as a homotetramer. We showed that TaGINS and TaMCM physically interact with each other, and this interaction probably represents the formation of part of a complex analogous to eukaryotic CMG complex for replication fork progression. In contrast to the case of *P. furiosus,* TaGINS has no effect on the helicase activity of TaMCM. The fact that the TaGINS inhibits the ATPase activity of TaMCM indicates that TaMCM undergoes a conformational change by interacting with TaGINS. However, the "MG complex" is not stable enough to work as a helicase, and another factor should be necessary for the functional helicase core complex (Fig. 7). It is interesting that Cdc6-2, one of the two Cdc6/Orc1 homologs in *T. acidophilum,* stimulates the helicase activity of TaMCM (Haugland et al. 2006). Further analyses showed that TaCdc6-2 enhances the ATPase activity of TaMCM probably by changing the conformation in the

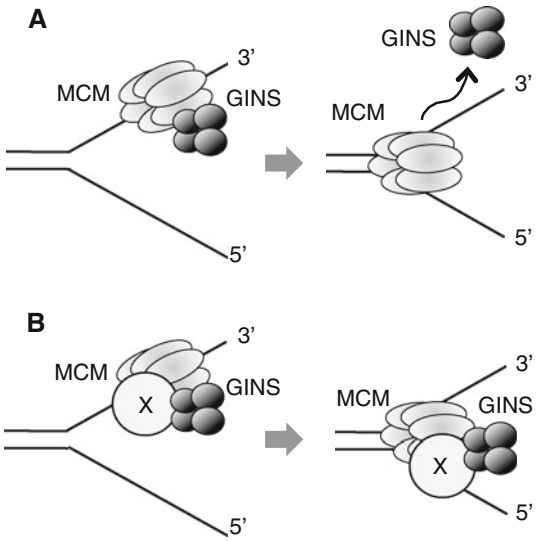

Fig. 7 Putative model for the interaction between TaGINS and TaMCM. **a** TaGINS interacts with TaMCM to decrease the ATPase activity. However, their interaction is not stable enough to affect the helicase activity of TaMCM as shown in the present in vitro studies. **b** TaGINS and TaMCM form a stable complex in the presence of another factor (X), possibly Cdc6-2, other unknown protein, or both of them, for replication fork progression, as a genuine helicase in *T. acidophilum* cells

TaMCM complex (Haugland et al. 2008b). Therefore, it should be investigated how the Cdc6 proteins are involved in the regulation of replication fork progression in *T. acidophilum.* One more important molecule should be considered for the helicase complex by analogy with the eukaryotic CMG complex. However, there is no sequence homolog for Cdc45 in the archaeal genomes in the current databases, and thus the identification of the functional counterpart of Cdc45 with a diverse sequence is now a very attractive subject in the research field of archaeal DNA replication. Protein modification, including phosphorylation, should also be investigated to consider the regulation mechanisms of the MCM function.

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