GINS Is a DNA Polymerase ε Accessory Factor during Chromosomal DNA Replication in Budding Yeast*§

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GINS is a protein complex found in eukaryotic cells that is composed of Sld5p, Psf1p, Psf2p, and Psf3p. GINS polypeptides are highly conserved in eukaryotes, and the GINS complex is required for chromosomal DNA replication in yeasts and Xenopus egg. This study reports purification and biochemical characterization of GINS from Saccharomyces cerevisiae. The results presented here demonstrate that GINS forms a 1:1 complex with DNA polymerase ε (Pol ε) holoenzyme and greatly stimulates its catalytic activity in vitro. In the presence of GINS, Pol ε is more processive and dissociates more readily from replicated DNA, while under identical conditions, proliferating cell nuclear antigen slightly stimulates Pol ε in vitro. These results strongly suggest that GINS is a Pol ε accessory protein during chromosomal DNA replication in budding yeast. Based on these results, we propose a model for molecular dynamics at eukaryotic chromosomal replication fork.

Three structurally and functionally distinct DNA polymerases, known as DNA polymerase α (Pol α), DNA polymerase δ (Pol δ), and DNA polymerase ε (Pol ε), act sequentially in yeast genomic DNA synthesis. The role of Pol δ in chromosomal DNA replication is well understood in both budding and fission yeasts (11, 12), and several lines of evidence support a role of Pol ε in processive synthesis of leading and lagging strand DNA (13–15). These observations suggest models for chromosomal DNA replication in which Pol ε and Pol δ play leading strand-and lagging strand-specific roles during chromosomal DNA replication, respectively. Pol ε has been proposed as the leading strand DNA polymerase because Pol ε is a highly processive polymerase without PCNA (1, 8) and pol3 mutants have defects in maturation of Okazaki fragments (10). Nevertheless, it has been reported that the N-terminal portion of budding yeast Pol ε (Pol2p), which includes motifs required for DNA polymerase and exonuclease activities, is dispensable for DNA replication, DNA repair, and viability (20, 21). However, this conclusion is controversial, because other studies suggest that deletion of the N-terminal region of Pol ε confers temperature sensitivity for growth, a defect in DNA elongation, premature senescence, and short telomeres. Furthermore, this pol2p deletion is lethal in combination with temperature-sensitive cdc2 and with exonuclease-deficient Pol δ (pol3-01). These results suggest that Pol ε plays a crucial role in maintaining genomic integrity (22, 23).

In a previous study, various sld (synthetic lethality with *dbp11-1*) mutants (24) were identified as yeast mutants that are lethal in combination with temperature-sensitive *dbp11-1* (25).
DPB11 encodes Dpb11p, a yeast protein that interacts with Pol ε that is required for initiation of chromosomal DNA replication. Sld1p is identical to Dpb3p, the second subunit of Pol ε. Sld2p binds to Dpb11p, and the Sld2p-Dpb11p heterodimer facilitates loading of Pol α-primase and Pol ε onto replication origins during S phase (26). Sld2p is phosphorylated by S-phase cyclin-dependent protein kinase at the beginning of S phase, and is required for initiation of chromosomal DNA replication (27). Sld3p interacts with Sld4p, which is identical to Cdc45p. Previous studies show that Cdc45p is required for initiation and elongation of chromosomal DNA replication (reviewed in Ref. 2). It has been reported that Sld3 is also important for the progression of DNA replication forks after the initiation step (28), as are Cdc45. In contrast, it does not move with DNA replication forks and only associates with MCM in an unstable manner as are Cdc45. In contrast, it does not move with DNA replication forks from early origins, it is no longer essential for the completion of chromosome replication (29). Sld5p is a component of GINS, which also includes Psf1p, Psf2p and Psf3p. GINS is essential for chromosomal DNA replication in yeast (30, 31). Finally, Sld6p is identical to Rad53p, which is required for cell cycle checkpoints (32). GINS associates with replication origins during S phase (30, 31). It has been suggested that GINS promotes an interaction between the Sld3p-Cdc45p complex and the Sld2-Dpb11-Pol ε, and that this could facilitate initiation of DNA replication (27). GINS polypeptides are conserved in eukaryotic cells. Xenopus egg extracts have a complex that resembles GINS, which is also required for chromosomal DNA replication (33). The X. laevis GINS complex consists of a structure that resembles a DNA clamp for Pol ε (33).

This study reports purification of GINS from yeast cell extracts. GINS, like other Pol ε clamp loading factors, it forms a 1:1 complex with Pol ε in an ATP-dependent catalytic activity in vitro. In the yeast cell, GINS is processive and dissociates most rapidly as a GINS ring during chromosomal DNA replication.

**EXPERIMENTAL PROCEDURES**

Yeast Strains—The yeast strains used in this study are YTS28 (MATα ura3–52 trpl-1122 pbr1-104 pep4-7 leu2-3, 112 nuc1Δ::LEU2 bar1::hisG), YTS28 (MATα ura3–52 trpl-1122 pbr1-104 pep4-7 leu2-3, 112 nuc1Δ::LEU2 bar1::hisG 6FLAG-PSF1::URA3), YTK61 (MATα ade2-1 bar1Δ::hisG can1-100 his3-11,15 leu2-3, 112 nuc1Δ::LEU2 bar1::hisG 6FLAG-PSF1::URA3), YTK62 (MATα ade2-1 bar1Δ::hisG can1-100 his3-11,15 leu2-3, 112 6FLAG-PSF1::URA3 GALp-CDCl1(TRP1) trpl-1 ura3-1). Other yeast strains are previously described (8, 22, 23, 30).

**Template Primer DNA—Oligonucleotides used in this study (Gene Design, Inc.) were purified by polyacrylamide gel electrophoresis. The 34-nucleotide primer (5′-CTAGTTACA GAGTTATGGTGACGATAAAAATCT-3′) was 32P-labeled at the 5′-end using [γ-32P]ATP and T4 polynucleotide kinase and annealed to the 65-mer (3′-GATCATAGTCTCTCAATACC ACTGCTATGTGATATCTCGCTAATGATATGATGTAATCTTAAGT-5′) oligonucleotide template to make a replication substrate for DNA polymerase assays.**

**Construction of Singly Primed Linear φX174 Single-stranded DNA—φX174 viral ssDNA (New England Biolabs) was annealed with a 30-mer oligonucleotide (5′-AGCGATAAAACTTGAGGTGGGTTAGCGCC-3′; a PstI site is underlined), and digested with the restriction endonuclease PstI to make a ssDNA. Then, a 30-mer primer (5′-TGCAAGGTGATACGGCAATCATTATTTATC-3′) was annealed to φX174 ssDNA as published (34).**

**Purification of GINS—Yeast strain YTS28 was grown in 60 liters of YPD (1% yeast extract, 2% polypeptide, 2% glucose) medium at 30 °C to about 2 × 108 cells/ml, harvested and stored at −80 °C until used. Cells (450 g) were thawed in 2 volume of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol) containing 1% (v/v) protease inhibitor mixture (Sigma). Cell disruption was achieved by a French press (2). The supernatant, which contained more than 90% of GINS complex, was applied to a 6 bed volumes of buffer A containing 150 mM NaCl, dialyzed against the same buffer and centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was applied to a Mono Q column (HR16/10, GE). After washing the column with buffer A containing 150 mM NaCl, proteins were eluted with a linear gradient from 150 to 600 mM NaCl in buffer A without 2-mercaptoethanol. 6× FLAG-tagged Psf1p was eluted at 350 mM NaCl in Buffer A. Fractions containing 6× FLAG-tagged Psf1p were pooled, supplemented with bovine serum albumin (BSA) to a final concentration of 5 mg/ml and with a mixture of protease inhibitors consisting of 4-(2-aminoethyl)-benzensulfonyl fluoride, aprotinin, benzamidine hydrochloride, leupeptin, and pepstatin A to final concentrations of 1 mM, 2 μg/ml, 1 mM, 10 μg/ml, and 1 μg/ml, respectively, and loaded to a 400 μl of anti-FLAG M2 agarose bead column. The column was washed three times with 10 bed volumes of buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.05% (v/v) Tween20, 0.005% (v/v) Nonidet P-40, 2 mM β-glycerophosphate, and 2 mM NaF) containing 300 mM NaCl and 0.1 mg/ml BSA, three times with 10 bed volumes of buffer B containing 300 mM NaCl, and once with 4 bed volumes of buffer B containing 150 mM NaCl and 10 μg/ml 1× FLAG peptide (Sigma). Bound proteins were eluted in 6 bed volumes of buffer.
B containing 300 mM NaCl and 100 μg/ml 3× FLAG peptide (Sigma). Eluted fractions were combined, applied to a Mono Q column (HR5/5, GE), the column was washed with 5 ml of 100 mM NaCl in Buffer A, and 6× FLAG-tagged Psf1p was eluted with 10 ml of a linear gradient from 0 to 700 mM NaCl in buffer A. Fractions containing 6× FLAG-Psf1p were pooled, dialyzed against buffer G (50% glycerol, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 3 mM 2-mercaptoethanol (50 μg/ml total 1 ml) and frozen in liquid nitrogen.

Pol ε and Other Replication Proteins—Pol ε holoenzyme was purified as described previously (8, 34). The Dpb3p-Dpb4p and Pol2p-Dpb2p complexes were kindly provided by Dr. S. Maki (Nara Institute of Science and Technology) (35). Purification of S. cerevisiae Pol δ, PCNA, and RPA was previously described (36). Pol α-primase was the same as described (8). Recombinant S. cerevisiae RF-C-1DN (37) was a gift of Dr. P. Burgers (Washington University).

Pol ε-GINS Binding Assay—The purified Pol ε holoenzyme was incubated with purified GINS or with FLAG-tagged GINS in binding mixtures consisting of 50 mM HEPES-KOH pH 7.6, 5 mM MgOAc, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 0.1 mg/ml BSA at 4 °C for 1 h. For cross-linking experiments, after the incubation, 1 μl of 0.2 M dithiobis (succinimidylpropionate) (DSP) was added to a binding mixture, which was further incubated at 24 °C for 30 min. The reaction was quenched by addition of 5 μl of 1 M Tris-HCl, pH 7.5, and incubation at 24 °C for 5 min. The mixtures were layered onto a 5 ml of 15–40% glycerol density gradient in buffer containing 50 mM HEPES-KOH (pH 7.6), 5 mM MgCl2, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 50 mM KOAc and centrifuged in a Hitachi P55ST2 rotor at 45,000 rpm for 14 h at 2 °C. The sample was fractionated into 23 fractions from top of the gradient. Proteins in the fractions were separated by SDS-PAGE, detected by silver staining or immunoblotting. Pol ε-GINS interaction was also detected with anti-FLAG antibody and anti-Psf1p antibody. 9× Mpf-FLAG and anti-Myc antibody (28).

DNA-binding oligonucleotide hybrids were made from Santa Cruz Biotechnology and were detected with anti-Myc and anti-Dpb4p rabbit polyclonal antibodies (22), and anti-yeast Pol II (22), -RPA (27), -SSB (25), and anti-Sld2p rabbit polyclonal antibody, which was expressed in E. coli.²

Immune precipitation—Without Cross-linking—The GINS holoenzyme was incubated with 3× FLAG-tagged Psf1p and GINS 6×-ligated DNA fragments were incubated with rabbit anti-Psf1p, anti-Psf2p, anti-Psf3p, anti-Dpb2p, anti-Cdc45p, and anti-Sld2p antibodies, respectively. Purified Psf1p, Psf2p, Psf3p, Dpb2p, Cdc45p, and Sld2p were immunoprecipitated from yeast cell extracts. Immune precipitates were boiled in SDS-sample buffer. Proteins associated with beads were detected by SDS-PAGE, followed by immunoblotting.

Immune precipitation of 6× FLAG-Psf1p from Yeast Cell Extracts—6× FLAG-tagged Psf1p was immunoprecipitated with anti-FLAG M2 agarose beads (Sigma) from the whole cell extracts prepared from asynchronously growing or hydroxyurea (HU)-treated yeast YYK61 cells as previously described (24). The precipitates were boiled in SDS-sample buffer at 95 °C for 30 min and subjected to SDS-PAGE, followed by immunoblotting.

Glycerol Gradient Sedimentation—Purified GINS and Pol ε (molar ratio was 1:1) were incubated in 100 μl of binding mixtures consisting of 50 mM HEPES-KOH pH 7.6, 5 mM MgOAc, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.1 mg/ml BSA at 4 °C for 1 h. For cross-linking experiments, after the incubation, 1 μl of 0.2 M dithiobis (succinimidylpropionate) (DSP) was added to a binding mixture, which was further incubated at 24 °C for 30 min. The reaction was quenched by addition of 5 μl of 1 M Tris-HCl, pH 7.5, and incubation at 24 °C for 5 min. The mixtures were layered onto a 5 ml of 15–40% glycerol density gradient in buffer containing 50 mM HEPES-KOH (pH 7.6), 5 mM MgCl2, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 50 mM KOAc and centrifuged in a Hitachi P55ST2 rotor at 45,000 rpm for 14 h at 2 °C. The sample was fractionated into 23 fractions from top of the gradient. Proteins in the fractions were separated by SDS-PAGE, detected by silver staining or immunoblotting. Pol ε-GINS interaction was also detected with anti-FLAG antibody and anti-Psf1p antibody. 9× Mpf-FLAG and anti-Myc antibody (28).

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² H. Araki, unpublished results.
as described (34). The GINS complex (600 fmol), Pol ε (800 fmol) and the 32P-labeled DNA fragment (60:15)(600 fmol) were incubated in reaction mixtures consisting of 20 mM HEPES-KOH (pH 7.6), 5 mM MgOAc, 0.05% Nonidet P-40, 10% glycerol, 0.1 mg/ml BSA, 0.1 mM dATP, and 0.1 mM dCTP at 22 °C for 5 min. The samples were cross-linked by addition of formaldehyde and DSP at final concentrations of 0.1% and 2 mM, respectively, and incubated at room temperature for 10 min. The samples were run on 4% polyacrylamide gels in TBE containing 5 mM MgOAc. The gels were fixed and dried, autoradiographed, and analyzed by Bio-Imaging Analyzer BAS-1800 (Fuji film).

Stimulation of Pol ε by GINS on a Replication Substrate Consisting of a 65-mer Template Annealed with 5′-32P-labeled 34-Nucleotide Primer—The 10-μl reactions contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 mM potassium glutamate, 0.1 mg/ml BSA, 1 mM dithiothreitol, 50 μM each dNTPs, 600 fmol of 5′-32P-labeled primer/template, 25–100 fmol of Pol ε, and 500 fmol of GINS. After incubation at 30 °C for 1–30 min, the reactions were stopped with 7 μl of 95% formamide-20 mM EDTA and electrophoresed on a 15% denaturing polyacrylamide gel containing 7 M urea.

RESULTS

GINS Is Part of the Yeast Replisome—Protein-interacting partners of GINS were identified by immunoprecipitation of FLAG-tagged Psf1p from logarithmically growing cells with anti-FLAG antibody beads. Immunoprecipitates were prepared in the presence or absence of a cross-linking agent, a small fraction of soluble Dpb2p (the second subunit of Pol ε) and Sld3p were detected. These results suggested that Dpb2p and Sld3p were binding to GINS. The identity of each GINS subunit was confirmed by immunoblotting purified GINS with anti-FLAG- and anti-Sld5p- antibodies (data not shown). The purity of GINS was estimated to be greater than 90% as determined by SDS-PAGE, followed by Western blotting. The purified flag-tagged Psf1p was also purified from asynchronously growing cells expressing 6× FLAG-tagged Psf1p (30) were incubated with (+) or without (−) formaldehyde and whole cell extracts (WCS) were prepared as described previously (24). 6× FLAG-tagged Psf1p was immunoprecipitated from the whole cell extracts with anti-FLAG beads. Proteins bound to the beads were released by boiling in SDS, and analyzed by SDS-PAGE, followed by immunoblotting with the indicated antibodies. WCS were also analyzed by SDS-PAGE, followed by immunoblotting with the indicated antibodies. In the figure, Dpb2p and Dpb3p were immunoprecipitated with anti-FLAG beads. In the immunoprecipitate containing cells expressing 3× FLAG-tagged Psf1p, Dpb2p was collected by centrifugation, but not any cross-linking reagent. 3× FLAG-tagged Psf1p with anti-FLAG antibody and anti-Dpb2 antibodies were analyzed by SDS-PAGE followed by immunoblotting with anti-Dpb2 antibodies. WCS were prepared as described previously (24) (Fig. 1A).

GINS Is An Accessory Protein of Pol ε—The above results confirmed that purified GINS is composed of Sld5p, Psf1p, Psf2p, and Psf3p. Purified GINS did not have any detectable ATPase-, ssDNA-binding-, or dsDNA binding activity (data not shown).

Pol ε was also purified from S. cerevisiae wild-type CB001 yeast cells as previously published (8, 34) and analyzed by SDS-PAGE, followed by immunoblotting. The purified Pol ε consisted of 4 subunit polypeptides, Pol2p, Dpb2p, Dpb3p, and Dpb4p, which were recognized by anti-Pol II*, anti-Dpb3*, and anti-Dpb4 antibodies, as previously described (8, 34).

GINS Specifically Binds to Pol ε—The physical interaction between GINS and Pol ε was examined using FLAG-tagged Psf1p and anti-FLAG antibody pull-down assays. When GINS and Pol ε were incubated at 25 °C for 30 min, anti-FLAG antibody beads coprecipitated FLAG-tagged Psf1p (GINS) and the Pol ε including Pol2p, Dpb2p, Dpb3p, and Dpb4p (Fig. 3A). GINS appeared to bind to Pol2p-Dpb2p as efficiently as Pol ε holoenzyme (Fig. 3B), but bound much less efficiently to Dpb3p-Dpb4p (35) (Fig. 3C) and to the 145 kDa-degradation product of the full size Pol2p (265 kDa), which has a DNA
polymerization activity as Pol ε (34) (data not shown). However, GINS did not bind to other replicative polymerases, Pol δ or Pol α-primase complex (Fig. 3D and data not shown for Pol α-primase). These results suggest that GINS interacts specifically with the C-terminal-half portion of Pol2p (a catalytic subunit of Pol ε) and possibly with Dpb2p, which is the second subunit of S. cerevisiae Pol ε. The C-terminal-half portion of Pol2p, where other subunits (Dpb2p, Dpb3p, and Dpb4p) of Pol ε interact and form Pol ε holoenzyme (1), is known to be essential for yeast cell growth (20, 21).

The interaction between GINS and Pol ε was also examined by glycerol density gradient sedimentation in the presence or absence of a protein cross-linking agent. GINS and Pol ε co-sedimented through the gradient, when the samples were pre-incubated with a cross-linking agent, but the complex dissociated spontaneously during sedimentation in the absence of a protein cross-linker (Fig. 4A). These results suggest that the interaction between GINS and Pol ε is relatively weak and/or transient. In contrast, GINS and Pol ε are stable as independent complexes in the absence of protein cross-linking during glycerol density gradient sedimentation (Fig. 4A). The glycerol density gradient data of their apparent molecular weights also suggests that GINS and Pol ε exist as monomers in solution and that they form a 1:1 GINS-Pol ε complex (Fig. 4B).

Purified GINS did not exhibit any DNA binding activity in the absence of Mg2+, where Pol ε readily bound single-stranded-, double-stranded-, 3'-tailed double-stranded-, 5'-tailed double-stranded-, Y-fork-like-, and bubble-structure DNA (35) (Fig. 5, A and B), or in the presence of Mg2+ (data not shown). On the other hand, Pol ε-GINS significantly bound to 5'-tailed double-stranded DNA and caused a further gel shift from that of Pol ε alone in the presence of Mg2+, although the gel shift was enormous, but was not as much as the Pol ε-DNA gel shift (Fig. 5C). When the polyclonal antibody raised against complex-bound Pol ε, a further gel shift was observed (right lane). This suggests that disruption of the complex by antibody treatment is specific to binding to GINS. In any case, to our knowledge, this is the first case that binding of eukaryotic DNA polym-

FIGURE 3. The GINS complex interacts with Pol ε, but not with Pol δ in vitro. A, fixed amount of purified Pol ε was incubated with increasing amounts of purified GINS containing 6×FLAG-tagged Psf1p (FLAG-GINS) or with FLAG-tagged BAP (FLAG-BAP) at 25 °C for 30 min. The samples contained 2.0 pmol of Pol ε and 0, 0.3, 0.5, or 1.0 pmol of GINS or 0, 0.6, 1.2, or 2.4 pmol of FLAG-BAP. The GINS complex or BAP was immunoprecipitated with anti-FLAG beads, and the precipitated proteins were analyzed by SDS-PAGE, followed by immunoblotting with antibodies to FLAG and Pol ε holoenzyme (Pol II*). B and C, GINS complex interacts with the Pol2-Dpb2 subcomplex, but not with the Dpb3p-Dpb4p subcomplex of Pol ε in vitro. The samples contained 2 pmol of GINS and 2 pmol of Pol ε holoenzyme (Pol ε), 0.7 or 1.8 pmol of Pol2-Dpb2 (Pol2-Dpb2) (B), or 0, 2, 4, or 10 pmol of Dpb3-Dpb4 (C). Binding reactions were carried out at 25 °C for 30 min. The GINS complex was immunoprecipitated with anti-FLAG beads and analyzed as in A using anti-Pol II*, anti-Dpb3p, and anti-Dpb4p antibodies. D, GINS complex was incubated with increasing amounts of purified Pol δ. The GINS complex was immunoprecipitated with anti-FLAG beads, and the precipitated proteins were probed with antibody to Pol31p (Hys2p), a subunit of Pol δ (4).
erase and its clamp to a DNA substrate has been demonstrated by gel shift assay.

**GINS Stimulates DNA Synthesis Catalyzed by Pol ε on a Oligonucleotide Template Primer Substrate**—Because GINS specifically interacts with Pol ε in vitro, it seemed possible that GINS has an effect on the catalytic activity of Pol ε. This idea was tested by measuring synthesis of DNA catalyzed by Pol ε in the presence or absence of GINS using excess DNA substrate. For this experiment, the DNA substrate was a 65-mer oligonucleotide annealed to a 32P-labeled 34-mer primer, which was converted to a 65-mer double-stranded DNA product by a DNA synthesis fill-in reaction. In the absence of GINS, although the efficiency was rather low, the fill-in reaction was completed in less than 1 min at 30 °C, indicating that the rate of DNA synthesis was >0.5 n/sec (Fig. 6, A and B). However, the amount of a 65-mer product reached a plateau during the 3–5 min (10 fmol and 35 fmol DNA were synthesized by 25 fmol and 50 fmol Pol ε, respectively), suggesting poor release and/or

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**FIGURE 4.** GINS forms a 1:1 complex with Pol ε holoenzyme or both in vitro.

GINS, Pol ε holoenzyme or both were incubated with 2 mM DSP (not cross-linked) and 15–40% glycerol gradient. The gradient was analyzed by SDS-PAGE, followed by fluorography. Positions of markers are indicated at the top. Panel a, distribution of Pol2p and Psf1p in A, each band of the markers was densitometry-scanned and estimated the relative intensity to the strongest band.

**FIGURE 5.** Gel shift assay of DNA binding activity by Pol ε or GINS in the absence of Mg2+.

Either Pol ε (A) or GINS (B) and various Cy5-labeled DNA fragments were incubated in reaction mixtures consisting of 20 mM HEPES-KOH (pH 7.6), 0.05% Nonidet P-40, 10% glycerol, 2 mM EDTA, and 0.1 mg/ml BSA on ice for 10 min. Ficoll was added to the samples at a final concentration of 15%, and the samples were run on a 4% polyacrylamide gel in TBE. The images were detected using a fluorescent image analyzer FLA-3000 (Fuji film). Asterisks show the position of Cy5 label on the DNA. C, gel-shift assay was carried out using GINS (0.6 pmol), Pol ε (0.8 pmol), GINS (0.6 pmol)-Pol ε (0.8 pmol), or GINS (0.6 pmol)-Pol ε (0.8 pmol)-FLAG antibody (10 pmol) and a 32P-labeled partially double-stranded 60-mer oligonucleotide DNA substrate (1.1 pmol) and + and − indicate with or without indicated proteins.

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*WITHDRAWN*
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FIGURE 7. GINS stimulates DNA synthesis catalyzed by Pol ε on a single-stranded circular template. DNA synthesis by Pol ε in the presence and absence of GINS (supplemental Figs. S2 and S3). A, reaction mixtures were the same as in A, except for 100 fmol of a 30-mer singly primed holoenzyme and 50 μm each dNTPs (Fig. 6, A and B). B, reaction mixtures were the same as in A, except for 100 fmol of a 30-mer singly primed holoenzyme and 50 μm each dNTPs (Fig. 6, A and B). C, reaction mixtures were the same as in A, except for 100 fmol of a 30-mer singly primed holoenzyme and 50 μm each dNTPs (Fig. 6, A and B). These results suggest that GINS stimulates DNA synthesis catalyzed by Pol ε on a single-stranded circular template. DNA synthesis on a single-stranded circular template was almost linear in the presence of GINS (Fig. 6A). Furthermore, total DNA products produced by Pol ε in the absence of GINS was two to three times longer than those products obtained in the presence of GINS than in the absence of GINS (Fig. 6A). However, the DNA products produced by Pol ε in the absence of GINS was two to three times longer than those products obtained in the presence of GINS (Fig. 6A). Because of the levels of incorporation during the incubation were less than 2% of the total incorporation obtained (only small portion of the input template primers were utilized), these results indicate that Pol ε starts elongation more or less uniformly and that the polymerase has a capacity to elongate DNA all the way around the viral DNA circle without dissociating from it in the presence or absence of GINS. These results suggest that GINS stimulates either the processivity or rate of DNA synthesis catalyzed by Pol ε or it stimulates both. Rates of DNA elongation by Pol δ and Pol ε with GINS, calculated from the maximum size of the elongation products at the first three time points, were about 15 and 30 nucleotides/s, respectively. After 5 min in the time course, the overall rate of DNA synthesis decreased gradually.

FIGURE 6. GINS stimulates DNA synthesis by Pol ε. A, Pol ε holoenzyme (25 or 50 fmol) was incubated with 600 fmol of DNA substrate (a 32P-labeled 34-nucleotide primer annealed to a 65-mer oligonucleotide template) with or without GINS (200 fmol) at 30 °C. Reaction products were analyzed on a 15% denaturing polyacrylamide gel, followed by autoradiography for 60 min or 18 h. B, results shown in A were quantified by measuring radioactivity of the 65-mer product. C, reactions were carried out and analyzed as in A except that the ratio of GINS to Pol ε was varied as indicated, and reactions were incubated for 5 or 10 min at 30 °C. D, PCNA and RF-C also stimulates DNA synthesis catalyzed by Pol ε. 25 fmol of Pol ε holoenzyme was incubated with 600 fmol of DNA substrate and 600 fmol of RF-C with or without various amounts of PCNA (0.8, 1.6, and 8 pmol) for 1, 5, and 10 min at 30 °C. The products were analyzed as in A, except for autoradiography for 12 h.
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This may be caused simply by a slowdown of the rate of DNA synthesis. And, these rates were estimated to be about 8 and 20 nucleotides/s, respectively. These are considerably slower than the estimated 35 nucleotides/s rate of replication fork movement in vivo in *S. cerevisiae* (22).

Although Pol ε holoenzyme itself is able to bind a singly primed dX174 ssDNA and GINS does not bind either ssDNA or dsDNA, GINS-Pol ε holoenzyme complex would have some difficulty to bind it, due to the predicted structure of GINS (33). Thus, above experiments were repeated using a singly primed dX174 sslDNA coated with yeast RPA. As shown in Fig. 7B, GINS also stimulated DNA synthesis by Pol ε as much as with dX174 ssDNA. Therefore, it is concluded that GINS-Pol ε complex is able to bind ssDNA as efficiently as it binds sslDNA. The *in vitro* DNA synthesis reactions catalyzed by Pol ε or Pol ε-GINS were not further stimulated by addition of PCNA and RF-C (Fig. 7C). These results suggest that GINS is an accessory protein for Pol ε and stimulates the Pol ε rate and possibly processivity of DNA synthesis in the absence of DNA polymerase clamp, PCNA, and its loader, RFC, *in vitro*.

**DISCUSSION**

This study shows that purified GINS forms a 1:1 complex with Pol ε, but not other replicases, (such as Pol δ or Pol α-primase), and that GINS stimulates Pol ε-catalyzed DNA synthesis *in vitro*. In the presence of GINS, the rate and possibly processivity of DNA synthesis catalyzed by Pol ε increased (Figs. 6 and 7). This stimulation is dependent on the ratio of GINS to Pol ε and also on the ratio of the substrate (Fig. 6C), unlike other Pol ε replicases. Thus, we concluded that GINS binds to Pol ε and Pol ε-GINS complexes can catalyze DNA synthesis in vitro. GINS is known to bind Pol ε readily, but the association of Pol ε with GINS is different from the association of Pol ε with PCNA. Pol ε-GINS complexes may have a 5-fold excess over that of Pol ε alone as the products were rather uniform in size during the incubation (Fig. 7). Thus, it could be roughly estimated that the processivity of Pol ε-GINS is ≈5.3 kb/binding event *in vitro*. Interestingly, Pol ε recycles many times during *in vitro* DNA synthesis on a linear small template primer in the presence of GINS, but not in its absence (Fig. 6 and supplementary Fig. S1). These reactions were partially substituted by PCNA and RF-C (Fig. 6D). These characteristics suggest that GINS may increase the suitability of Pol ε for catalyzing leading strand DNA synthesis *in vivo*. Thus, the data presented here are consistent with the proposal that GINS is a Pol ε accessory factor and that Pol ε-GINS plays a significant role as a leading strand DNA polymerase.

Previous studies showed that GINS associates with origins of replication and adjacent DNA sequences in yeast DNA (30, 31) and that GINS facilitates association of Dpb11p and Cdc45p with chromatin (30). Genetic and physical interaction studies also suggested that GINS interacts with Sld3p and Dpb11p and that Sld3p and Dpb11p facilitate binding of GINS to origins of replication (30). This study provides evidence that GINS is a part of the yeast replisome *in vivo* (Fig. 1) and that GINS directly interacts with Pol ε *in vitro* (Figs. 3 and 4). Other groups also showed recently that GINS is one of many replication proteins found at the replication forks (40, 41). Particularly, it has been shown that all three Pol s, α, δ, and ε, Mcm2-7p, Cdc45p, GINS, and Mcm10p are found in the vertebrate replisome. Furthermore, in the presence of the DNA polymerase inhibitor aphidicolin, which causes uncoupling of a highly processive DNA helicase from the stalled replisome, only Cdc45p, GINS, and Mcm2-7p, but not any of Pol s, are enriched at the pause site (41). These results are consistent with our finding of a weak and/or transient association of GINS to Pol ε holoenzyme. Therefore, we suggest that Dpb11p, Sld3p, Cdc45p, GINS, and Pol ε assemble in a coordinate manner on replication origins prior to initiation of DNA synthesis *in vivo*.

One of the functions of PCNA is to recruit Pol δ onto a primer site and to increase the processivity of Pol δ during Okazaki fragment synthesis on lagging strand DNA (reviewed in Ref. 34). We showed recently that PCNA and RF-C greatly stimulate both Pol ε and Pol δ-catalyzed elongation of a short oligonucleotide primer site and to stimulate the processivity of Pol ε (Fig. 7A and B), but Pol α-catalyzed elongation in the absence of GINS (Fig. 7C). Previous studies (40, 41) suggested that all three Pols, α, δ, and ε, were reconstituted and purified from insect cells (33), and also showed that Pol ε-holoenzyme primarily utilizes GINS as an accessory factor for DNA synthesis *in vitro* and *in vivo*.

Based on results from our previous studies (30) and the present article, we propose the following model to explain how Pol ε-GINS complex binds a template-primer DNA during DNA synthesis and how both leading- and lagging strand synthesis are achieved by three different DNA polymerases at eukaryotic chromosomal replication forks. Recently, we observed that *Xenopus* GINS, which was reconstituted and purified from insect cells (33), stimulates
GINS is an Accessory Protein of Pol ε

DNA synthesis catalyzed by Xenopus Pol ε (Fig. 5). In any case, if Pol ε-primase was a clamp for Pol ε, Pol ε-primase on a leading strand at origin, is a component of the pre-RC during M and G1 phase, and is a helicase that is predicted to unwind DNA at the replication fork. Consequently, the replication fork movement, but not the initiation timing, is retarded in pol2-16 mutant cells, which express a DNA polymerase domain less Pol ε-polypeptide and are presumed that the leading strand synthesis is substituted with Pol ε-primase (40). Thus, Dpb11p and Cdc45p, along with Pol ε-primase and RPA bound to the looping single-stranded DNA, although we could not rule out a possibility that PCNA also binds on the leading strand in this study, PCNA is omitted from the figure. Previous studies indicate that Dpb11p and Sld2p are loaded onto replication origin, but they do not bind their specific roles during eukaryotic chromosomal DNA replication.

DNA synthesis catalyzed by E. coli DnaB. DnaB becomes highly active when coupled to Pol III holoenzyme, and this coupling occurs through the γ-subunit of the clamp loader (45). If this analogy can be applied to eukaryotic systems, then we propose that MCM helicase activity is somewhat reminiscent of the relatively weak helicase of M. tuberculosis DNA replication. Thus, this model is consistent with the finding that Pol ε, which has capability of both ds- and ssDNA binding (34, 35), as a tether to the DNA template, although there is no evidence to indicate the RNA primer synthesized by Pol α-primase.

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