Sulfolobus Replication Factor C Stimulates the Activity of DNA Polymerase B1

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Replication factor C (RFC) is known to function in loading proliferating cell nuclear antigen (PCNA) onto primed DNA, allowing PCNA to tether DNA polymerase for highly processive DNA synthesis in eukaryotic and archaeal replication. In this report, we show that an RFC complex from the hyperthermophilic archaea of the genus Sulfolobus physically interacts with DNA polymerase B1 (PolB1) and enhances both the polymerase and 3′-5′ exonuclease activities of PolB1 in an ATP-independent manner. Stimulation of the PolB1 activity by RFC is independent of the ability of RFC to bind DNA but is consistent with the ability of RFC to facilitate DNA binding by PolB1 through protein-protein interaction. These results suggest that Sulfolobus RFC may play a role in recruiting DNA polymerase for efficient primer extension, in addition to clamp loading, during DNA replication.

Materials and Methods

Substrates. Oligonucleotides (see Table S1 in the supplemental material) were synthesized at Sangon BioTech (Shanghai, China). To prepare a primed template, a primer was labeled at the 5′ end using T4 polynucleotide kinase (TaKaRa) and [γ-32P]ATP (PerkinElmer). The labeled primer was purified using a G25 microspin column (GE Healthcare) and annealed to a template at a molar ratio of 1:1.5. Annealing reactions were carried out in 20 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Plasmid pUC18 containing a single nick was prepared by DNase I cleavage in the presence of ethidium bromide as described previously (13).

Protein purification. Expression plasmids for S. solfataricus PolB1, RFCα, and RFCβ were constructed and transformed into E. coli strain Rosetta(DE3)/pLysS (Novagen), and the recombinant proteins were prepared as described previously (6, 14). Protein concentrations were determined by the Lowry method using bovine serum albumin (BSA) as the standard.

Site-directed mutagenesis. A quadruple mutant of RFCβ containing alanine substitutions at Arg84, Arg90, Thr120, and Lys149 was constructed by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene) (for mutagenesis primers, see Table S1 in the supplemental material). The expression plasmid for RFCαRFCβ was used as the template in the mutant construction. The mutations were

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verified by DNA sequencing. The recombinant mutant protein was purified as described for the wild-type protein.

**Strain construction.** A *Sulfolobus islandicus* strain that synthesized PolB1 as a hybrid protein containing a Strep tag and a His<sub>6</sub> tag in succession at its C terminus (PolB1-Tag) was constructed by employing a double-crossover-based recombination method (15). DNA fragments containing the C-terminal coding sequence of the polB1 gene (SisRe_1451) and a polB1 downstream flanking sequence were made by PCR from the *S. islandicus* genomic DNA, using primers listed in Table S1 in the supplemental material. A sequence encoding Strep and His<sub>6</sub> was included in polB1-strep-his<sub>6</sub>-pyrEF-polB1 downstream region, into plasmid pUC18. The replacement plasmid was propagated in *Escherichia coli*, isolated, linearized, and transformed into *S. islandicus* E234, as described previously (16). Transformants were selected by colony growth at 75°C on plates containing basic salts supplemented with 0.2% Casamino Acids, 0.2% sucrose, 0.05% yeast extract, and a vitamin mix (16). Colonies were picked and inoculated in a liquid medium of the same composition. After growth to the late exponential phase at 75°C, cells were harvested, and the genomic DNA was extracted as described previously (17). The desired gene replacement was confirmed by PCR and sequencing.

**Pulldown assays.** *S. islandicus* (PolB1-Tag) was grown to the exponential phase, and the cells were harvested and resuspended in 50 mM Tris-HCl (pH 8.0) and 10% (wt/vol) glycerol. After sonication on ice, the lysate was centrifuged at 12,000 × g for 30 min at 4°C. Ni-nitriol triacetic acid (NTA) beads (5% of the sample volume; GE Healthcare) were added to the supernatant, and the sample was shaken gently for 30 min at 10°C. The beads were recovered by centrifugation and washed six times with 1 ml of wash buffer (50 mM Tris-HCl [pH 8.0], 40 mM imidazole, and 10% [wt/vol] glycerol) and twice with 1 ml of DNase I buffer. For DNase I treatment, the beads were resuspended in DNase I buffer in a final volume of 50 µl. DNase I (350 U; TaKaRa) was added, and incubation was carried out for 30 min at 37°C. Both DNase I-treated and untreated samples were again washed six times with 1 ml of wash buffer. The beads were recovered and boiled in sample buffer for SDS-PAGE, and the proteins retained by the beads were subjected to electrophoresis in a 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were electro photorechemically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and detected by immunoblotting with anti-RFC<sub>B</sub> rabbit antibodies.

**Blue native PAGE (BN-PAGE).** PolB1 (30 pmol) and/or RFC (30 pmol) was precipitated for 30 min at 37°C in 20 mM Bis-Tris- HCl (pH 7.0), 0.1 M 6-aminocaproic acid, 5 mM MgCl<sub>2</sub>, and 7% (wt/vol) sucrose. The samples were electrophoresed at 10°C through a 5 to 15% gradient blue native polyacrylamide gel as described previously (18). Following electrophoresis, proteins were detected by staining with Coomassie brilliant blue G250 or by immunoblotting.

**BlI.** Bio-layer interferometry (BL1) was performed at 30°C with a speed of 1,000 rpm on an Octet Red system (Forto Bio) according to the manufacturer’s instructions. To detect the interaction between RFC and PolB1, PolB1 was immobilized onto amine-reactive (AR) biosensors, whereas the indicated amounts of RFC were in buffer A (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 4% [wt/vol] glycerol, 1% BSA, and 0.5% Tween 20). To determine the binding of RFC to DNA, 5'-biotinylated pUC18 (see Table S1 in the supplemental material), to which pUC18 (Table S1) was annealed, was immobilized onto streptavidin (6) biosensors, and the indicated amounts of RFC were in buffer A supplemented with 2 mM MgCl<sub>2</sub>. The biosensors were first immersed in the RFC solution for 300 s to allow RFC to interact with PolB1 or the DNA and were subsequently dipped in buffer A for 300 s to allow protein dissociation. The kinetic rate constants <i>k<sub>i</sub></i> and <i>k<sub>d</sub></i> and the equilibrium dissociation constant <i>K<sub>D</sub></i> were derived using a 1:1 binding model as described in the manufacturer’s instructions.

**Polymerase assays.** The standard polymerase assay mixtures (20 µl) contained 50 mM Tris-HCl (pH 8.0), 2 mM β-mercaptoethanol, 100 µg/ml BSA, 4 mM MgCl<sub>2</sub>, 4 mM PolB1, 4 nM<sup>32</sup>P-labeled DNA substrate, 1 mM deoxy nucleoside triphosphates (dNTPs), and the indicated amounts of RFC. ATP or ATP-γ-S (1 mM) was added when indicated. Following incubation for 15 min at 65°C, the mixture was extracted with phenol-chloroform and mixed with an equal volume of 2× loading buffer (95% deionized formamide, 100 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol FF for denaturing polyacrylamide gel electrophoresis or 100 mM NaOH, 10 mM EDTA, 6% Ficoll 400, 0.025% bromocresol green, and 0.025% xylene cyanol FF for alkaline agarose gel electrophoresis). After boiling for 3 min and subsequent cooling on ice, the sample was subjected to electrophoresis in a 15% denaturing polyacrylamide gel containing 8 M urea in 1× Tris-borate-EDTA (TBE) or in a 1.2% alkaline agarose gel in 50 mM NaOH and 1 mM EDTA. The gel was dried and exposed to X-ray film or analyzed using an ImageQuant Storm PhosphorImager (GE Healthcare).

**Exonuclease assays.** The standard exonuclease assay mixtures (20 µl) contained 50 mM Tris-HCl (pH 8.0), 2 mM β-mercaptoethanol, 100 µg/ml BSA, 4 mM MgCl<sub>2</sub>, 4 nM PolB1, 4 nM<sup>32</sup>P-labeled DNA substrate, and the indicated amounts of RFC. Following incubation for 15 min at 65°C, the mixture was extracted with phenol-chloroform and mixed with an equal volume of 2× loading buffer (95% deionized formamide, 100 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol FF). After boiling for 3 min and cooling on ice, the sample was subjected to electrophoresis in a 15% denaturing polyacrylamide gel containing 8 M urea in 1× TBE. The gel was dried and exposed to X-ray film or analyzed using an ImageQuant Storm PhosphorImager (GE Healthcare).

**Electrophoretic mobility shift assays (EMSA).** PolB1 and/or RFC was incubated for 10 min at 23°C with 2 nM<sup>32</sup>P-labeled DNA in 20 mM Tris-HCl (pH 8.0), 2 mM β-mercaptoethanol, 0.1 mM EDTA, and 100 µg/ml BSA. The mixtures were subjected to electrophoresis in an 8% polyacrylamide gel in 0.1× TBE. The gel was exposed to X-ray film or analyzed using an ImageQuant Storm PhosphorImager (GE Healthcare).

**Binding of RFC to nicked pUC18.** Wild-type or mutant RFC (5 pmol) was mixed with singly nicked pUC18 (5 pmol) in 20 mM Tris-HCl (pH 8.0), 8 mM MgCl<sub>2</sub>, 5 mM EDTA, 0.1 mM EDTA, 80 µg/ml BSA, and 4% (wt/vol) glycerol in a final volume of 50 µl. ATP-γ-S (4 mM) was added when indicated. After incubation for 10 min at 70°C, the mixture was applied to a G50 microspin column (GE Healthcare), in which the G50 resin was replaced with Sepharose CL-4B (Sigma). The column was pre-equilibrated in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM DTT, 0.5 mM EDTA, 50 µg/ml BSA, and 3% (wt/vol) glycerol. Following centrifugation for 2 min at 400 × g, fractions containing the RFC–DNA–ATP-γ-S complex, eluted in the void volume, were collected. An aliquot (10 µl) of each fraction was subjected to electrophoresis in a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred to a PVDF membrane and detected by immunoblotting with anti-RFC<sub>B</sub> rabbit antibodies.

**RESULTS**

RFC interacts with PolB1. Replication proteins act in a highly controlled and efficiently coordinated fashion at the fork of DNA replication. To learn more about the proteins involved in the fork movement, we have conducted a systematic survey of complex interactions among these proteins in *S. islandicus* using an approach involving epitope tagging. Both *S. islandicus* and *S. solfataricus*, two closely related *Sulfolobus* species, were used in the present study. We chose *S. islandicus* for the tagging experiment because a highly efficient genetic system has been developed for this species (15). Since PolB1, RFC<sub>B</sub>, and RFC<sub>C</sub> from the two species are nearly identical, with 96, 92, and 96% homology, respec-
tively, at the amino acid sequence level, the three proteins from one species are believed to behave in the same manner as those from the other species. PolB1 from *S. islandicus* was among the replication proteins that we selected for the screen. First, we constructed *S. islandicus* PolB1-Tag, an *S. islandicus* strain in which the PolB1 gene was replaced with a gene encoding the polymerase containing a Strep-His10 tag at the C terminus. The *S. islandicus* PolB1-Tag cells were grown to the exponential phase, harvested, and lysed. The cell extract was subjected to pulldown assays. Strikingly, RFC (RFCs) is hereinafter referred to as RFC) appeared to be readily pulled down with PolB1 (Fig. 1A). The same result was obtained when the sample was treated with DNase I after the pull-down step, eliminating the possibility that RFC was pulled down with PolB1 as a result of their interaction with DNA. As a control, no RFC was pulled down when the cell extract of the original *S. islandicus* strain, from which *S. islandicus* PolB1-Tag was constructed, was used in the assay. Furthermore, no subunits of the heterotrimeric PCNA were found to be associated with the beads, suggesting that RFC was not pulled down with PolB1 by interacting with PCNA (see Fig. S1 in the supplemental material).

To confirm the RFC-PolB1 interaction, we performed a BN-PAGE assay using the recombinant form of the two proteins. As shown in Fig. 1B, the RFC complex (201 kDa) alone existed as a single or double heteropentamer, as judged by molecular masses of the corresponding bands. Similarly, PolB1 (101 kDa) alone was found predominantly as monomers and dimers. When the two proteins were mixed, new protein bands appeared, suggesting the formation of RFC-PolB1 complexes of various combinations (e.g., 1 RFC/1 PolB1 and 1 RFC/2 PolB1). The coexistence of both proteins in the new bands was verified by immunoblotting (data not shown). It has been reported that PolB1 was able to bind DNA as a trimmer (19). Whether a dimeric form of PolB1 or RFC is of physiological relevance remains to be understood. It is worth noting that the ability of RFC to form a complex with a PolB1 dimer suggests that the binding site of PolB1 for RFC differed from that for the self-association of PolB1 into a dimer.

We then determined quantitatively the RFC-PolB1 interaction by bio-layer interferometry (BLI). The dissociation constant \( K_D \) of the RFC-PolB1 interaction was calculated to be 0.4 \( \mu \)M (Fig. 1C). Based on the above results, we conclude that RFC is capable of interacting physically with PolB1.

RFC stimulates the polymerase and 3′-5′ exonuclease activities of PolB1. The observation that RFC interacted with PolB1 prompted us to investigate if RFC would affect the activities of PolB1. First, we examined the influence of RFC on the polymerase activity of PolB1. As shown in Fig. 2A, extension of a primer annealed to an oligonucleotide template (p42/t76) by PolB1 was significantly enhanced by RFC. For example, primer extension by 4 nM PolB1 was twice as efficient in the presence of 10 nM RFC as in the absence of RFC. The maximum stimulation of polymerization by PolB1 (4 nM) was achieved when RFC was added at \( \approx \) 0.2 \( \mu \)M, as expected from the \( K_D \) of the RFC-PolB1 interaction. We also tested the effect of RFC on the ability of PolB1 to extend a primer on an M13 single-stranded DNA (ssDNA) template (p42/M13mp19) (Fig. 2B). The extension products synthesized by PolB1 increased markedly both in maximum size and in quantity in the presence of RFC compared to those in the absence of RFC. It is worth noting that the stimulation of the PolB1 activity by RFC was also observed when the template DNA was bound by replication protein A (RPA) (see Fig. S2 in the supplemental material).

Since RFC is an ATPase, we sought to determine if ATP binding or hydrolysis was required for the stimulation of PolB1 activity by RFC. We detected no significant differences in the stimulation of DNA polymerization by PolB1 in the presence and the absence

**FIG 1** Interaction between RFC and PolB1. (A) Pulldown analysis of the interaction between RFC and PolB1 from *S. islandicus*. *S. islandicus* PolB1-Tag was grown to the exponential phase and harvested. The cell extract was incubated with Ni\(^{2+}\) beads. The beads were subsequently washed and treated with DNase I, if indicated. Proteins retained by the beads were subjected to SDS-PAGE and identified by immunoblotting with antibodies against RFCs. Lane C, a cell extract from *S. islandicus* instead of *S. islandicus* PolB1-Tag, was used in the assay as a control for nonspecific binding of RFC to Ni\(^{2+}\) beads. (B) Blue native PAGE, RFC (30 pmol) and PolB1 (30 pmol) were mixed individually or in combination with loading buffer for blue native PAGE. Samples were subjected to electrophoresis in a 5 to 15% gradient blue native polyacrylamide gel at 10°C. Molecular mass markers are indicated on the left. RFC-PolB1 complexes are indicated by arrows. (C) Bio-layer interferometry. Amine-reactive (AR) biosensors, covered with immobilized PolB1, were treated in RFC solutions of increasing concentration (160, 320, 650, 1,300, and 2,600 nM), as represented by curves from bottom to top, respectively. The rate constants \( k_a \) and \( k_d \) were determined, and the equilibrium dissociation constant \( K_D \), was calculated as described by the manufacturer.
of 1 mM ATP (Fig. 2C). Besides, addition of ATP-γ-S, a nonhydrolyzable analog of ATP, did not reduce the ability of RFC to stimulate the PolB1 activity. Since RFC might be able to use dATP, which was present in the reaction mixture, as the substrate (13), we tested a nucleotide-binding mutant of RFC in the assay. The mutant RFC was also capable of stimulating the polymerase activity of PolB1 (see Fig. S3 in the supplemental material). These results indicate that neither binding nor hydrolysis of ATP or any other nucleotide was required for the stimulatory effect of RFC on the PolB1 activity.

Next, we examined the effect of RFC on the exonuclease activity of PolB1. As a highly active 3′-5′ exonuclease, PolB1 degraded both ssDNA and double-stranded DNA (dsDNA) with similar efficiencies at 65°C (14). As shown in Fig. 3, cleavage of 32P-labeled p42, whether it was single stranded or annealed to a complementary strand (t42 or t76), by PolB1 was substantially enhanced in the presence of RFC. However, the addition of RFC did not appear to alter the pattern of p42 cleavage by PolB1.

Since RFC is a heteropentamer of a large subunit (RFC4) and four identical small subunits (RFC5), we sought to determine if the RFC subunit was able to stimulate the PolB1 activity. Unfortunately, our repeated attempts to overproduce RFC4 alone in Escherichia coli were unsuccessful, as has been experienced by others (6, 8). Therefore, we tested only the effect of RFC5 on the PolB1 activity. RFC5 alone formed a hexamer (data not shown) (20, 21). As shown in Fig. 4A, addition of as much as 75 nM RFC5 resulted in no significant change in the amount of extension by PolB1 (2 nM). By comparison, primer extension in the presence of RFC5 alone was nearly 30-fold higher than that in the absence of RFC5. Similarly, the exonuclease activity of PolB1 was not enhanced by RFC5 (Fig. 4B). Taken together, our results suggest that heteropentameric RFC was capable of stimulating both the polymerase and the 3′-5′ exonuclease activities of PolB1.

RFC facilitates DNA binding by PolB1 through protein-protein interaction. To understand how RFC might stimulate the activities of PolB1, we first tested if the stimulation depended on
the ability of RFC to bind DNA. In other words, we wanted to examine the possibility that RFC interacted with template DNA, increasing its availability for binding or DNA synthesis by PolB1. We prepared a mutant RFCS defective in DNA binding by substituting alanine for each of the four conserved positively charged residues, i.e., Arg84, Arg90, Thr120, and Lys149, and assembling an RFCSL pentamer with the mutated RFCS. It was reported that counterparts of these residues were oriented toward the central chamber in the yeast RFC–PCNA–ATP–γ-S structure (22) and that mutation of these conserved residues reduced DNA binding by yeast RFC as well as E. coli RFCS complex (23, 24). We then compared wild-type and mutant RFC in DNA binding by bio-layer interferometry. As shown in Fig. 5A, wild-type RFC bound the primed template p42/t76 with a $K_D$ of 20 nM, an affinity similar to that of yeast RFC (23), whereas the mutant complex lost the ability to bind the DNA (Fig. 5B). In addition, the mutant RFC was unable to bind ssDNA (see Fig. S4 in the supplemental material).

Taking advantage of the fact that RFC is able to form more stable complexes with DNA in the presence of ATP–γ-S, a nonhydrolyzable analog of ATP, than in its absence (25), we also examined the ability of wild-type and mutant RFC to bind to singly nicked pUC18 by gel filtration (Fig. 5C). Wild-type RFC formed a large protein-pUC18 complex in the presence of ATP–γ-S, whereas mutant RFC was unable to bind the nicked pUC18. Furthermore, mutant RFC was unable to load PCNA onto nicked plasmid, and its ATPase activity was not stimulated by DNA (data not shown).

These results confirmed that the ability to bind DNA was drastically reduced in mutant RFC. Interestingly, however, mutant RFC was similar to the wild-type complex in stimulating the activity of PolB1 (Fig. 5D, E, F, and G). Therefore, we conclude that stimulation of PolB1 activity by RFC was independent of the ability of RFC to bind DNA.

It is also possible that RFC increased the affinity of PolB1 for template DNA by interacting physically with the polymerase. We tested this possibility by studying the effect of RFC on the binding of PolB1 to template DNA by EMSA. As shown in Fig. 6A, wild-type RFC generated no detectable shifts on the primed template p42/t76 under the experimental conditions used. This was not surprising since it was previously observed that the interaction of RFC with DNA was detectable by EMSA only when they were fixed with glutaraldehyde (25). PolB1 bound p42/t76 with an apparent dissociation constant of 80 to 160 nM, as estimated based on the amount of the protein required to retard half of the input DNA. Binding of PolB1 to p42/t76 generated three resolved shifts (Fig. 6A). The first two shifts suggest a single p42/t76 molecule bound by one and two PolB1 molecules or, presumably, a PolB1 monomer and a PolB1 dimer, respectively. The top shift, located near the position of the sample loading well, may have resulted from binding of multiple PolB1 molecules to the DNA and/or protein-DNA aggregates of unknown structures. Under the assay condi-

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**Figures:**
- **Fig 3** RFC stimulates the exonuclease activity of PolB1. (A) Effect of RFC on DNA cleavage by PolB1. PolB1 (4 nM) was incubated with $^{32}$P-labeled p42, p42/t42, or p42/t76 (2 nM) in the presence of the indicated amounts of RFC for 15 min at 65°C. Samples were extracted with phenol-chloroform and subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea in 1× TBE. The gel was exposed to X-ray film. Left and right panels, reactions were performed at the same time and run on two separate gels under identical conditions. p42, p42/t42, and p42/t76 are shown as ssDNA, dsDNA, and p/tDNA, respectively. (B) Quantitative analysis. Samples, prepared as described above, were subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea in 1× TBE. DNA cleavage was quantified using a phosphorimager. The data shown represent averages of three independent measurements.
- **Fig 4** Effect of RFCS on the activities of PolB1. (A) Effect of RFCS on primer extension by PolB1. PolB1 (2 nM) was incubated for 15 min at 65°C with $^{32}$P-labeled p42/t76 (2 nM) in the presence or absence of RFCS or RFCSL (75 nM). Samples were extracted with phenol-chloroform, and subjected to electrophoresis in a 15% polyacrylamide gel containing 8 M urea in 1× TBE. The gel was exposed to X-ray film. Lanes from the same gel are juxtaposed, as indicated by a thin black line. (B) Effect of RFCS on DNA cleavage by PolB1. PolB1 (2 nM) was incubated for 15 min at 65°C with $^{32}$P-labeled p42/t76 (2 nM) in the presence or absence of RFCSs or RFCSL (75 nM). Samples were processed as described above.
tions, where ~12% of the input DNA was retarded, forming primarily the first shift, in the presence of PolB1 alone, addition of RFC led to a significant increase in the amount of PolB1-DNA complexes and the accumulation of the second shift (Fig. 6A). Interestingly, although RFC promoted DNA binding by PolB1, no changes in mobility between shifts generated in the presence of RFC and those generated in the absence of RFC were detected. This implies that no RFC was stably bound to the PolB1-DNA complexes under the assay conditions used. Formation of PolB1-DNA complexes was similarly promoted when mutant RFC defective in DNA binding, instead of wild-type RFC, was used in the assay, suggesting that the RFC effect on DNA binding by PolB1 was independent of the DNA-binding ability of RFC (see Fig. S5 in the supplemental material). Furthermore, the ability of RFC to

FIG 5 Stimulation of the PolB1 activity by RFC is independent of the ability of RFC to bind DNA. (A and B) Bio-layer interferometry. Streptavidin biosensors, covered with immobilized 5'-biotinylated t76 annealed to p42, were treated with wild-type (WT) or mutant (MUT) RFC solutions of increasing concentration (20, 39, 78, 156, and 312 nM), as represented by curves from bottom to top, respectively. The rate constants $k_a$ and $k_d$ were determined, and the equilibrium dissociation constant $K_D$ was calculated as described by the manufacturer. (C) Binding of singly nicked pUC18 by RFC. Wild-type or mutant RFC (5 pmol) was mixed with singly nicked pUC18 (5 pmol) in the presence of ATP-γ-S (4 mM). Following incubation for 10 min at 70°C, the mixture was applied to a modified G50 column. Fractions in the void volume were collected, and an aliquot of each fraction was subjected to SDS-PAGE, followed by immunoblotting with anti-RFC antibodies. Lane C, positive control. (D) Comparison of the effects of wild-type and mutant RFC on primer extension by PolB1. PolB1 (4 nM) was incubated for 15 min at 65°C with 32P-labeled p42/t76 (4 nM) in the presence of the indicated amounts of wild-type or mutant RFC. Samples were extracted with phenol-chloroform and subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea in 1× TBE. The gel was exposed to X-ray film. (E) Quantitative analysis. Samples, prepared as described for panel D, were subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea in 1× TBE. The gel was analyzed using a phosphorimager. The data shown represent averages of three independent measurements. (F) Comparison of the effects of wild-type and mutant RFC on the exonuclease activity of PolB1. PolB1 (4 nM) was incubated for 15 min at 65°C with 32P-labeled p42/t76 (4 nM) in the presence of the indicated amounts of wild-type or mutant RFC. Samples were processed as described for panel D. (G) Quantitative analysis. Samples, prepared as described for panel F, were subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea in 1× TBE. The gel was analyzed using a phosphorimager. The data shown represent averages of three independent measurements.
enhance DNA binding by PolB1 did not depend on the presence of a template primer, since similar observations were made when p42/t76 was replaced with p42 or oligo(dT)42 in the assay (Fig. 6C and data not shown). The second shift was absent when the 42-nucleotide (nt) ssDNA was used in the assays, probably because the DNA was not long enough to accommodate two PolB1 molecules. These results indicate that the RFC-PolB1 interaction facilitated the binding of template DNA by PolB1. Intriguingly, when assays were performed with ≥150 mM NaCl, the interaction of RFC with PolB1 diminished, and so did the stimulatory effect of RFC on DNA binding by and polymerase activity of PolB1 (data not shown). Therefore, it appears likely that RFC stimulated the activities of PolB1 by promoting template DNA binding by the polymerase.

**DISCUSSION**

In addition to its primary role in loading a sliding clamp onto template DNA, permitting processive DNA synthesis by replicative DNA polymerase, RFC has also been shown to serve additional functions in DNA replication. Human RFC stimulates the nuclease activity of Flap endonuclease 1 in an ATP-independent manner (26) and inhibits DNA joining by DNA ligase 1, whose phosphorylation regulates the interaction between the two proteins (27–29). *S. solfataricus* RFC interacts with eukaryotic-type primase (PriSL), resulting in modulation of the activities of both proteins (10). In this report, we show that RFC interacted with PolB1 with an estimated $K_d$ of 0.4 μM. This value is lower than the intracellular RFC concentration, which is about 2.4 μM based on an estimate of ~1,500 molecules of RFC in a cell (see Fig. S6 in the supplemental material) and assuming an intracellular volume of 1 μm$^3$. Therefore, it appears that the observed RFC-PolB1 interaction in vitro is of physiological relevance. We found that RFC stimulated the polymerase and 3’-5’ exonuclease activities of the polymerase in an ATP-independent manner. Similar observations were made earlier on RFC from the crenarchaeon *Aeropyrum pernix*, which significantly enhanced primer extension by Pol I and Pol II, two family B DNA polymerases (30). More recently, it was reported that the rate of nucleotide incorporation by PolB1 on a primed template at 60°C in the presence of RFC was nearly twice as high as that in the absence of the clamp loader in the *S. solfa-
taricus system (31). However, RFC from the euryarchaeon Methanobacterium thermoautotrophicum ΔH slightly inhibited DNA synthesis by PolB (13). Clamp loaders from the euryarchaeon Methanosarcina acetivorans and Archaeoglobus fulgidus did not appear to affect DNA synthesis in the absence of PCNA (7, 32). The reason for the discrepancy is unknown but may relate to differences in the architecture of their replication apparatus or to the experimental conditions under which the effect of RFC on DNA polymerase was assayed. The multifunctional roles of RFC are probably required for coordination of the functions of the various components of a replisome during the postpriming stage of DNA replication.

S. solfataricus RFC enhanced the activity of PolB1 by increasing template binding by the polymerase, probably through protein-protein interactions. The interactions between the clamp loader complex and DNA polymerase appear to be of functional importance in Bacteria and Eukarya. In E. coli, the clamp loader γ complex possesses three β subunits, each of which contacts a Pol III core, allowing coordinated DNA synthesis on the leading and lagging strands by three molecules of Pol III core (33–35). In humans, the fate of RFC after PCNA loading remains to be established. Some suggested that RFC disengages from DNA after PCNA loading (36–38), whereas others found that RFC remains attached to the primer terminus and moves along the DNA with PCNA and Pol6 through chain elongation (39, 40). The latter scenario appears to be consistent with the finding that RFC interacted with FEN1 and ligase I, two proteins participating in Okazaki fragment maturation (26, 27). As it moves along the DNA during the fork progression, RFC would be readily available for stimulating FEN1 in flap cleavage and participating in the sealing of nicks between Okazaki fragments. In Sulfolobus, the ability to interact with PolB1 may allow RFC to play a role essential for high-efficiency DNA replication. PolB1 has been shown to be a distributive DNA polymerase with an estimated processivity of only 25 to 30 nucleotides, even when anchored to the DNA template by PCNA (31). The polymerase frequently disengages from the template during DNA synthesis, leaving PCNA on the template. Therefore, PolB1 molecules are continuously recruited throughout the process of DNA replication. Conceivably, RFC may serve to recruit PolB1, permitting efficient polymerase exchanges in the replisome.

The ability of RFC to interact with both primase (10) and PolB1 raises the possibility that it serves a role in the switch from primer synthesis to primer extension, or the transfer of a primer from primase to PolB1, during DNA replication. We speculate that RFC binds to and opens the PCNA ring while promoting the release of primase from the template DNA. RFC then loads PCNA onto the DNA at the position of a nascent primer. PolB1 is bound by RFC, instead of PCNA, prior to the completion of PCNA loading. After PCNA is loaded onto the DNA, PolB1 recruited by RFC is transferred to PCNA, and high-fidelity DNA synthesis ensues.

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