The Replication Factor C Clamp Loader Requires Arginine Finger Sensors to Drive DNA Binding and Proliferating Cell Nuclear Antigen Loading**

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Replication factor C (RFC) is an AAA+ heterodimer that couples the energy of ATP binding and hydrolysis to the loading of the DNA polymerase processivity clamp, proliferating cell nuclear antigen (PCNA), onto DNA. RFC consists of five subunits in a spiral arrangement (RFC-A, -B, -C, -D, and -E, corresponding to subunits RFC1, RFC4, RFC3, RFC2, and RFC5, respectively). The RFC subunits are AAA+ family proteins and the complex contains four ATP sites (sites A, B, C, and D) located at subunit interfaces. In each ATP site, an arginine residue from one subunit is located near the γ-phosphate of ATP bound in the adjacent subunit. These arginines act as “arginine fingers” that can potentially perform two functions: sensing that ATP is bound and catalyzing ATP hydrolysis. In this study, the arginine fingers in RFC were mutated to examine the steps in the PCNA loading mechanism that occur after RFC binds ATP. This report finds that the ATP sites of RFC function in distinct steps during loading of PCNA onto DNA. ATP binding to RFC powers recruitment and opening of PCNA and activates a γ-phosphate sensor in ATP site C that promotes DNA association. ATP hydrolysis in site D is uniquely stimulated by PCNA, and we propose that this event is coupled to PCNA closure around DNA, which starts an ordered hydrolysis around the ring. PCNA closure severs contact to RFC subunits D and E (RFC2 and RFC5), and the γ-phosphate sensor of ATP site C is switched off, resulting in low affinity of RFC for DNA and ejection of RFC from the site of PCNA loading.

All cellular organisms utilize a multiprotein ATP-driven DNA replicase, which functions with other proteins to duplicate chromosomal DNA prior to cell division (1). DNA replicases are composed of a DNA polymerase, a ring-shaped processivity clamp, and a clamp loader ATPase (reviewed in Ref. 2).

The DNA polymerase binds to the processivity clamp protein, which completely encircles DNA. The clamp slides on DNA and is pulled along by the polymerase during chain extension, continuously holding polymerase to DNA and constraining it to act in a highly processive manner.

The eukaryotic ring-shaped processivity factor, proliferating cell nuclear antigen (PCNA), is a trimer of three identical subunits arranged head-to-tail to generate a ring with a large central cavity for encircling DNA (3, 4). PCNA confers processivity on DNA polymerase δ (5–7) and also interacts with other factors involved in DNA metabolism such as DNA polymerase ε, flap endonuclease-1, DNA ligase, mismatch repair proteins, and many others (8, 9). An interface between two PCNA monomers must be disrupted and opened to place DNA into the center of the ring. The ring must then be re-closed for the clamp to remain bound to the DNA and function with the polymerase. The eukaryotic clamp loader complex, replication factor C (RFC), uses the energy of ATP binding and hydrolysis to recruit the processivity clamp to DNA, break one clamp interface, and topologically link the clamp to primed template DNA (10–15). Studies in the *Escherichia coli* system have shown that the clamp loader and DNA polymerase compete for the clamp, and therefore the clamp loader must eject from the clamp for the DNA polymerase to use it (16). Likewise, RFC and Pol δ also compete for binding to PCNA (17, 18). Therefore, after PCNA is linked to DNA, RFC ejects from the clamp to allow the polymerase access to the clamp (19) (summarized in Fig. 1A).

A crystal structure of *Saccharomyces cerevisiae* RFC-ATPγS-PCNA reveals RFC to consist of a circular collar from which the five ATP-binding modules are suspended (Fig. 1B) (20). This subunit arrangement is similar to the *E. coli* γ complex clamp loader (21), which loads the ring-shaped β clamp onto DNA (22). The current study refers to the five RFC subunits based on their sequential order in the pentamer: RFC-A for RFC1, RFC-B for RFC4, RFC-C for RFC3, RFC-D for RFC2, and RFC-E for RFC5 (see Fig. 1C). This alphabetical nomenclature facilitates the comparison of subunits in analogous positions in RFC and γ complex.

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** The abbreviations used are: PCNA, proliferating cell nuclear antigen; RFC, replication factor C; ATPγS, adenosine 5′-O-(thiophosphoryl); ssDNA, single-stranded DNA; SRC, Ser-Arg-Cys; SSB, single-stranded DNA-binding protein; BSA, bovine serum albumin; DTT, dithiothreitol; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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The structural similarity between prokaryotic and eukaryotic clamps and clamp loaders suggests that the clamp loading mechanisms may be similar, in general terms, and biochemical studies in both systems support this suggestion (23–30). The clamp loader binds ATP to promote clamp binding and opening. The clamp loader-ATP-open clamp complex then associates with primed template DNA. The association of clamp loader-ATP-clamp complex with DNA does not appear to require ATP hydrolysis, and it is thought that DNA is positioned inside the central channel of the clamp during this step (20, 31–33). DNA binding triggers ATP hydrolysis in the clamp loader, which then closes the clamp and ejects from DNA, leaving the closed ring on DNA. This final stage of the clamp loading mechanism requires hydrolysis of ATP and is currently viewed as a single step, although multiple ATP molecules are hydrolyzed, and thus this stage of the reaction is likely composed of multiple steps.

The five RFC subunits each contain three domains (domains I–III), and the primary sequence of these domains is homologous among the five subunits; only the RFC-A (RFC1) subunit has additional N- and C-terminal regions (34, 35). The RFC pentamer complex is mainly held together by a tightly packed “collar” region consisting of domain III of each RFC subunit (see Fig. 1, A and B). The N-terminal ATPase modules, composed of domains I and II, are arranged in a right-handed spiral with an overall pitch that closely matches double-stranded DNA (20). A cavity exists in the center of this protein spiral that is sufficiently large to accommodate double-stranded DNA. Furthermore, conserved polar and positively charged residues line this cavity and are proposed to interact with DNA (20, 31). There is a gap between two subunits, RFC-A and RFC-E (illustrated in Fig. 1, A and C), which is presumed to provide an exit path for single-stranded DNA from the central cavity (20). Mutation of the conserved residues that line the central cavity in RFC causes a decrease in DNA binding, which supports the hypothesis that DNA binds in the central cavity of these clamp loaders (32). A similar study in RFC shows that residues within the central cavity are required for DNA binding (36).

A recent electron microscopic reconstruction of Pyrococcus furiosus RFC reveals that PCNA adopts a spiral conformation when held open in a complex of RFC-ATPγS-PCNA-DNA (37). Consistent with this observation, molecular dynamics simulations of yeast PCNA indicate that PCNA alone may readily adopt a right-handed spiral when open (38). The spiral conformation of open PCNA may allow a more intimate contact between the clamp and the five-subunit spiral of RFC.

The ATPase modules of the five RFC subunits are similar in structure to the AAA+ family of ATPases (39). This family includes a wide variety of factors that couple ATP binding and hydrolysis to remodel protein substrates (40). A common fea-

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**Figures:**

**A.** Schematic model of the ATP binding- and hydrolysis-coupled steps of PCNA loading. After PCNA is loaded, RFC must disengage from PCNA to allow the polymerase access to the clamp. B, crystal structure of RFC-ATPγS-PCNA (20). RFC is partially engaged with the closed PCNA ring. The C-terminal collar of RFC (top) is composed of domain III of each RFC subunit and domains I and II form the AAA+ modules that bind ATP and PCNA. C, right: schematic of the arrangement of ATP sites in the AAA+ modules of the RFC heteropentamer. Each ATP site is at a subunit interface. The neighboring subunit contains an arginine finger in a conserved SRC motif that interacts with the γ-phosphate of the ATP bound to the adjacent subunit. Left: schematic depicting ATP site C from the crystal structure of RFC-PCNA (left), ATP (black) bound to RFC-C (RFC3) interacts with the RFC-C P-loop (yellow). The SRC arginine finger (blue) of RFC-D is proximal to the γ-phosphate of the ATP.
The ATP Cycle of RFC

Previous studies of prokaryotic and eukaryotic clamp loaders suggest that individual ATP sites play specific roles in clamp loading (29, 44, 49). Mutational analysis in yeast RFC has shown that ATP binding to sites C and D of RFC is essential for DNA binding (29), but it is not clear how ATP binding is coupled to DNA binding. In E. coli γ complex, the arginine finger in ATP site D functions allosterically in promoting β clamp association, and the arginine fingers in sites B and/or C are involved in promoting DNA binding (44). These findings support the suggestion that there is a division of activities among ATP sites in clamp loaders. Perhaps specific ATP sites control other important clamp loader actions such as clamp closing and clamp loader ejection from the clamp and DNA.

This report examines different RFC complexes that have one or more arginine finger residues mutated to alanine. We show that none of the arginine fingers of RFC are needed for the steps of PCNA interaction and ring opening. However, our results demonstrate that certain ATP sites of RFC play distinct roles downstream of PCNA opening. The arginine finger in ATP site C is needed for RFC to bind DNA. ATP hydrolysis in site D is specifically triggered by PCNA, and we propose that hydrolysis in this site leads to closure of PCNA around DNA. The crystal structure of RFC-PCNA shows that closed PCNA no longer interacts with RFC-D and RFC-E, and thus ring closure is presumed to lead to loss of RFC affinity for PCNA and dissociation of RFC from the PCNA-DNA complex.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ADP and ATP were purchased from Sigma, unlabeled ATPγS and eosin-5-maleimide were from Roche Diagnostics, and radioactive nucleoside triphosphates were from PerkinElmer Life Sciences, Inc. The following proteins were purified as described: PCNA and PCNA (Cys81-Only) (50) and E. coli SS (51). PCNA and RFC concentrations were determined by absorbance at 280 nm in 6 M guanidinium hydrochloride using extinction coefficients calculated from the Trp and Tyr content (PCNA, ε280 = 5120 m−1 cm−1; RFC, ε280 = 158,880 m−1 cm−1). All other protein concentrations were determined by Bradford assay reagent (Bio-Rad) using BSA as a standard. PCNAHK is a PCNA construct with a His-tag and a N-terminal 6-residue kinase recognition site (52). PCNAHK was purified in one step by nickel chelate chromatography. PCNAHK was labeled to a specific activity of 100 dpm fmol with [γ-32P]ATP using the recombinant catalytic subunit of cyclin-dependent protein kinase produced in E. coli (a gift from Dr. Susan Taylor, University of California at San Diego). The following oligonucleotides were synthesized and gel-purified by Integrated DNA Technologies: 30-mer, 5′-CGATATCTGGGGCAGGTGCAGGAGGATTTCGCA3′; 66-mer, 5′-CCATTTGTTAACGCCAGGTTCAGCTGAACCGATCCAAAGCGAGCAGGTATTTGCGT3′. A 102-mer 3′-biotinylated oligonucleotide was synthesized and gel-purified by the W. M. Keck Facility at Yale University: 5′-CCATTTGTTAACGCCAGGTTCAGCTGAACCGATCCAAAGCGAGCAGGTATTTGCGT3′. Biotin was from PerkinElmer Life Sciences, Inc. The following proteins were purchased: Bio-Gel P-100, P-200, and P-400 (Bio-Rad); 30-mer DNA oligonucleotide as described (54). The Bio-Gel P-100 and P-200 columns were purchased from Schleicher and Schuell, and polyethyleneimine-cellulose TLC plates were purchased from EM Science. Streptavidin-coated Dynabead M-280 magnetic beads were purchased from Dynal Biotech.

Buffers—Buffer A is 30 mM Hepes-NaOH (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Buffer B is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Membrane Wash Buffer is: 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 150 mM NaCl. Reaction buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 4% glycerol (v/v), and 40 μg/ml BSA. Gel filtration buffer is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 10 mM MgCl₂, 100 μg/ml BSA, and 4% glycerol (v/v). Clamp loading buffer is 30 mM Hepes-NaOH.
The ATP Cycle of RFC

(pH 7.5), 7 mM MgCl₂, 130 mM NaCl, 1 mM DTT, 1 mM CHAPS. ATPase buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 10% glycerol (v/v).

Construction and Purification of RFC(SAC) Mutants—Individual RFC-B (RFC4), RFC-C (RFC3), RFC-D (RFC2), and RFC-E (RFC5) genes were mutated using the QuikChange method (Stratagene). The following mutations were made: RFC-B (RFC4) Arg₂⁵⁷ to Ala, RFC-C (RFC3) Arg₂⁶⁰ to Ala, RFC-D (RFC2) Arg₂⁸³ to Ala, RFC-E (RFC5) Arg₂⁸⁴ to Ala. All S. cerevisiae five-subunit RFC complexes used in this study were expressed in E. coli using a two-plasmid system described previously (55). In all RFC constructs, the first 283 residues of the RFC-A (RFC1) subunit were deleted and RFC-A contained an N-terminal His-kinase tag (52). All RFC complexes were harvested by centrifugation, resuspended in 100 ml of Buffer A containing 100 mM NaCl, and isopropyl β-D-thiogalactopyranoside was added to 0.5 mM, followed by a 12-h incubation with shaking at 15 °C. Cells were harvested by centrifugation, resuspended in 100 ml of Buffer A containing 150 mM NaCl, and lysed using a French press at 22,000 p.s.i. 2 mM PMSF was added to the cell lysate, and the lysate was clarified by centrifugation. The supernatant was applied to a 100 ml of SP-Sepharose (Amersham Biosciences) column equilibrated in Buffer A containing 150 mM NaCl. Protein was eluted in a 750-ml gradient from 150–600 mM NaCl.

The five-subunit RFC complex eluted at ~510 mM NaCl, separating from RFC subcomplexes that eluted in earlier fractions. Fractions from the SP-Sepharose column containing five-subunit RFC were pooled, diluted in Buffer B to a conductivity equal to 100 mM NaCl, and loaded onto a 15-ml Fast Flow Q-Sepharose column (Amersham Biosciences) equilibrated in Buffer B containing 90 mM NaCl. Protein was eluted using an 80-ml gradient of Buffer B from 90–500 mM NaCl. Fractions containing RFC eluted at ~250 mM NaCl and were pooled, aliquoted, and stored at ~80 °C. The protein yield was ~50 mg from 12 liters of E. coli culture.

ATPγS Binding Assays—Nitrocellulose membrane circles (25 mm) were washed with 0.5 mM NaOH, rinsed immediately with water, and equilibrated in membrane wash buffer before use. Reactions contained 1 mM RFC and [32S]ATPγS (0–25 μM) in Buffer B + 160 mM NaCl and 10 mM MgCl₂ in a total volume of 15 μl. After 1-min incubation on ice, 10 μl of the reaction was filtered through a pretreated nitrocellulose membrane on a glass microanalysis filter assembly (Fisher) at a rate of 60 μl/min. The membrane was immediately washed with 150 μl of ice-cold membrane wash buffer at ~1 ml/min. Membrane circles were dried, and radioactivity was measured by liquid scintillation counting. An apparent Kₚ was determined using the simple binding model, RFC + ATPγS ↔ RFC-ATPγS, to fit the data using KaleidaGraph (Synergy Software).

Clamp Loading Assays Using Primed M13mp18 ssDNA—Clamp loading was measured using [32P]-PCNAHK (29.4 nM as trimer), which was incubated for 10 min at 30 °C with RFC BCDE(SAC) or wild-type RFC (16.7 nM), in 60 μl of Reaction Buffer containing primed M13mp18 ssDNA (17 nM), SSB (4.2 μM as tetramer), 0.5 mM ATP, and 10 mM MgCl₂. The reaction was applied to a 5-ml Bio-Gel A-15m column (Bio-Rad) equilibrated in gel filtration buffer containing 100 mM NaCl at 23 °C. Thirty-five fractions of 180 μl each were collected, and 100 μl was analyzed by liquid scintillation. 32P-PCNAHK-DNA complex elutes in the early fractions (fractions 9–15), while the free 32P-PCNAHK elutes later (fractions 18–31). The amount of PCNA in each fraction was determined from its known specific activity.

Magnetic Bead PCNA Clamp Loading Assay—The 30-mer/102-mer biotinylated DNA was conjugated to Dynabeads M-280 Streptavidin (Dynal Biotech), and the DNA-bead conjugate was incubated with 5 mg/ml BSA in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl at room temperature for 30 min with agitation and washed three times in the same buffer until no BSA remained in the supernatant. Typical yield was ~100 pmol of DNA/mg of Dynabeads, as analyzed by comparing DNA ethidium bromide staining intensities in a polycrylamide gel using known amounts of primed DNA as a standard. Clamp loading reactions were performed at 16 °C in loading buffer. Mixtures containing final concentrations of 0.5 mM ATP, 170 nM DNA, 510 nM E. coli SSB (tetramer), 700 nM 32P-PCNAHK (trimer), but lacking RFC, were agitated by pipette and incubated for 1 min at 16 °C, then clamp loading was initiated upon adding RFC to a final concentration of 100 nM. Reactions were quenched with a final concentration of 21 mM EDTA (pH 7.5) at the indicated time points. Quenched reactions were placed on ice for 1 min, then the DNA beads were isolated with a magnetic concentrator (1 min) at 4 °C. Beads were washed twice in clamp loading buffer containing 300 mM NaCl at 4 °C. Protein was stripped from the beads using 1% SDS and counted by liquid scintillation.

PCNA Ring Opening Assay—The following residues in the S. cerevisiae PCNA gene were mutated by the QuikChange procedure (Stratagene): Cys²² to Ser, Cys³⁵ to Ser, Cys⁶² to Ser. One buried native cysteine residue (Cys⁸¹) at each trimer interface remained in this PCNA construct, referred to as PCNA (Cys⁸¹-Only). PCNA ring opening was tested by treatment with eosin-5-maleimide, a thiol-reactive dye. Ring opening reactions contained 20 mM Tris-HCl (pH 7.1), 200 mM NaCl, 0.5 mM EDTA, 10 mM MgCl₂, 100 μM ATPγS, 3 μM PCNA (Cys⁸¹-Only), 3 μM RFC, and 30 μM eosin-5-maleimide (final concentrations in 15 μl). Reactions were incubated without eosin-5-maleimide for 1 min on ice, initiated with eosin-5-maleimide, and quenched after 30 s with 5 μl of 1 M DTT. Control assays without RFC contained 1.1 mg/ml BSA. Quenched reactions were analyzed in the dark on a 10% SDS-polyacrylamide gel, and labeled PCNA was visualized in a UV scan (305 nm) on a Fluor-S Multilager (Bio-Rad).

Fluorescent DNA Binding Assays—RFC binding to DNA was measured using a fluorescently labeled primed template DNA. A synthetic 30-mer oligonucleotide, modified at the 3’-hydroxyl with a C6 spacer primary amino group, was reacted with a Rhodamine Red-X-NHS-ester (Integrated DNA Technologies) to form a Rhodamine Red-X-conjugated 30-mer. “Rh-P/T” primed template DNA was prepared by mixing 1.0 nmol of Rhodamine Red-X-conjugated 30-mer (Integrated DNA Technologies) with 1.2 nmol of unlabelled 66-mer in 100 μl of 5 mM Tris-HCl, 150 mM NaCl, 15 mM sodium citrate (final pH 8.5)
and annealed at 37 °C for 1 h. RFC complexes were titrated from
0–420 nm into reactions of 60 μl final volume containing 20
mM Tris-HCl (pH 7.5), 175 mM NaCl, 10 mM MgCl₂, 5 mM DTT, 0.5
mM EDTA, 100 μM ATPγS or ADP, and 35 nM Rh-P/T
DNA. When present, PCNA trimer was at a concentration of
810 nm. The fluorophore was excited at 570 nm, and a 580–680
nm scan was taken. Relative intensity of the peak at 588 nm was
plotted versus RFC concentration. Apparent $K_d$ measurements
were determined using a simple model (RFC + DNA ↔ RFC-
DNA) to fit the data using the KaleidaGraph program (Synergy
Software). Attempts to measure primer-template association by
a change in fluorescence anisotropy were complicated by a
low signal of RFC that bound nonspecifically to regions other
than the primer-template junction. Measurement of fluores-
cence intensity change of the probe at the primer-template
junction avoids the influence of this nonspecific binding activ-
ity. Mutation of DNA-interacting residues of RFC-B, -C, and -D
was performed as described previously (36).

**Steady-state ATP Hydrolysis of RFC(SAC) Mutants**—ATPase
assays contained 500 nm RFC, 1 mM [α-32P]ATP, 2 μM PCNA
trimer (when present), and 1 μM synthetic 30/66 DNA (when
present) in a final volume of 30 μl of ATPase buffer containing
150 mM NaCl. The synthetic primer/template DNA is linear
and allows PCNA to slide off the ends after it is loaded. Hence,
PCNA is continuously recycled during these assays. Reactions
were brought to 30 °C and initiated upon addition of RFC. Ali-
quots of 5 μl were removed at 1 min intervals from 0–3 min (for
“+DNA” and “+ PCNA+DNA” samples) or at 5 min intervals
from 0–20 min (for “alone” and “+ PCNA” samples) and
quenched with an equal volume of 0.5 M EDTA (pH 7.5). 1 μl of
each quenched aliquot was spotted on a polyethylenimine cel-
lulose TLC sheet (EM Science) and developed in 0.6 M potas-
sium phosphate buffer (pH 3.4). The TLC sheet was dried, and
[α-32P]ATP and [α-32P]ADP were quantitated using a Phos-
phorImager (GE Healthcare). Three independent experiments
were performed and the standard deviation is indicated in the
figure chart. A similar procedure was used when PCNA was
titrated into a fixed concentration of either wild-type RFC or
RFC E(SAC), except that each reaction was analyzed at a fixed 2
min time point.

**RESULTS**

**Arginine Finger Mutants of RFC Bind ATP Similarly to Wild
Type**—The N-terminal region of RFC-A, containing the first
283 residues, is not required for cell viability, displays nonspe-
cific DNA binding activity, and deletion of this region results in
a complex with higher specific activity than RFC containing
full-length RFC-A (56, 57). All RFC complexes used in this
study carry a deletion of this region. The RFC complex contain-
ing the truncated RFC-A with no additional point mutations to
any subunit is referred to herein as “wild-type” RFC.

Arginine to alanine mutations were constructed in each of the
four S. cerevisiae RFC subunits with SRC motifs. Five-sub-
unit complexes of RFC-A/B/C/D/E containing the mutation in
either RFC-B, RFC-C, RFC-D, or RFC-E were expressed in
E. coli using a two-plasmid system and purified as described
previously for wild-type RFC (55). These single mutant five-
subunit complexes are referred to as: RFC B(SAC), RFC
C(SAC), RFC D(SAC), and RFC E(SAC). RFC-A does not con-
tain an SRC motif. Therefore, no point mutants of RFC-A were
used in this study.

We also constructed a quadruple arginine finger mutant,
RFC BCDE(SAC) complex, which lacks arginine fingers
toentially. Residual activities inherent to RFC BCDE(SAC) may
be presumed to be independent of the arginine fingers. All of
the single RFC(SAC) mutants should therefore be able to pro-
gress through the PCNA loading mechanism at least as far as
the RFC BCDE(SAC) mutant. The RFC BCDE(SAC) complex
and the single RFC(SAC) complexes were expressed and puri-
fied similarly to wild-type RFC and have comparable appear-
ance in an SDS-polyacrylamide gel (Fig. 2A), suggesting that
the RFC(SAC) mutants fold properly.

Previous studies of the arginine fingers in the E. coli γ com-
plex clamp loader concluded that these residues do not contrib-
ute to ATP binding (43). To test whether this is true for the RFC
arginine fingers, we examined the ability of the RFC
BCDE(SAC) mutant to bind nucleoside triphosphate. Using
a nitrocellulose filter binding assay we determined the $K_d$
value of wild-type RFC for ATPγS to be ~3.3 μM. The RFC BCDE(SAC)
mutant displayed a $K_d$ for ATPγS of 5.5 μM, compa-
rable with wild-type RFC (Fig. 2B). This result indicates that
the arginine fingers of RFC do not significantly contribute to ATP
binding.

**The RFC BCDE(SAC) Complex Cannot Load PCNA**—To test
whether ATP binding to RFC can promote PCNA loading in
the absence of the arginine fingers, we measured the ability of
the RFC BCDE(SAC) mutant to recruit PCNA to a primer-
template DNA. A circular single-stranded M13mp18 phage
dNA, uniquely primed with a DNA oligonucleotide and coated
with E. coli SSB, was used as a substrate for PCNA loading.
To follow clamp loading on DNA we used a PCNA construct con-
taining a six-residue N-terminal kinase recognition sequence
A

**Efficient PCNA Loading**—We next addressed which arginine
fingers are required for RFC to load PCNA by testing single
RFC(SAC) mutants for PCNA loading activity. Since wild-type
RFC can load PCNA onto DNA in a very short time frame, we
designed a PCNA loading assay that allows rate measurements
over a 25-s time course (see scheme in Fig. 3). In this assay, a
synthetic primed template is first conjugated to a magnetic

![Figure 2A](image-url) **The ATP Cycle of RFC**
bead. To prevent PCNA sliding off the free end of the linear DNA, the template was designed with sufficient single-stranded DNA on both sides of the primer to facilitate the binding of *E. coli* SSB, which blocks PCNA from sliding off (51). 

32P-Labeled PCNA was added to the magnetic bead-coupled primed DNA, and clamp loading was initiated upon addition of RFC. Reactions were rapidly quenched at timed intervals upon adding EDTA and then placed at 4 °C. PCNA remains stably associated with the SSB-coated DNA with a half-life of ~50 min at 4 °C (data not shown). The magnetic bead-conjugated DNA template enabled rapid separation of PCNA-DNA complexes from free PCNA within 1 min. Western analysis showed that RFC does not remain bound to the PCNA-DNA complex (data not shown).

The single (SAC) mutants displayed a spectrum of PCNA loading rates (Fig. 3). The RFC B(SAC) mutant was comparable with wild-type RFC. The RFC C(SAC) mutant was moderately deficient, displaying ~35% the rate of clamp loading by wild-type RFC. In contrast, the RFC D(SAC) and RFC E(SAC) mutants were less than 5% the rate of wild-type RFC, suggesting that ATP sites C (location of the RFC-D(SAC) mutation) and ATP site D (location of the RFC-E(SAC) mutation) play important roles in driving PCNA loading.

The Arginine Finger Mutants of RFC Can Bind and Open PCNA

— Which steps in clamp loading are the arginine finger mutants of RFC deficient in? Do any of the steps occur prior to ATP hydrolysis? We used the non-hydrolyzable ATP analogue, ATPγS, to test the single RFC(SAC) mutants for a role prior to ATP hydrolysis. PCNA ring opening and DNA binding do not require ATP hydrolysis by RFC, and therefore we developed assays to examine these two steps.

First, we developed a PCNA opening assay based on a similar assay for ring opening that we used previously for the *E. coli* system (27, 58). We prepared a construct of PCNA in which the three surface-exposed cysteine residues were mutated to serine, leaving only one cysteine, Cys81, that is buried at each trimer interface (Fig. 4A). This “PCNA (Cys81-Only)” clamp is resistant to labeling by the thiol-reactive dye, eosin-5-maleimide.
The ATP Cycle of RFC

Next, we tested various RFC(SAC) mutants for ability to open PCNA in the presence of ATPγS. The single RFC(SAC) mutants were all active for PCNA opening (Fig. 4B, middle). In fact, even the RFC BCDE(SAC) mutant was capable of opening PCNA (Fig. 4B, bottom). Therefore the clamp loading deficiency of the RFC D(SAC) and RFC E(SAC) mutants (e.g. see Fig. 3) must be blocked at a step in the PCNA loading mechanism that occurs after PCNA opening.

A γ-Phosphate Sensor in ATP Site C of RFC Regulates DNA Binding—We next examined the RFC mutants for ability to bind DNA. ATP binding is known to promote RFC association with DNA, even in the absence of PCNA (28). To analyze DNA binding by RFC, we used a synthetic DNA with a fluorescent rhodamine derivative attached to the 3’ terminus of the primer strand (see scheme in Fig. 5). We refer to this template as Rh-P/T. A change in fluorescence intensity indicates association of RFC at the primer-template junction and we have shown previously that RFC binds this DNA substrate with an affinity comparable with that of unmodified DNA (28, 36).

In Fig. 5A, we measured the affinity for DNA of wild-type and arginine finger mutant RFC complexes by titrating RFC into a fixed concentration of Rh-P/T DNA in the presence of ATPγS. Wild-type RFC produces an increase in fluorescence intensity in this assay. Fig. 5B shows that ADP does not suffice to power the conformational change in wild-type RFC needed for DNA binding. The RFC C(SAC) and RFC B(SAC) mutants had affinities for the primed DNA that were equal or 2-fold reduced, respectively, compared with wild-type RFC; the RFC E(SAC) mutant displayed a 6-fold reduced affinity for the primed DNA. However, RFC D(SAC) lost essentially all detectable DNA binding in this assay ($K_d > 5 \mu M$). Therefore, the arginine finger of RFC-D is likely needed to recognize ATP in site C to promote the conformational change in RFC required for DNA binding.

The RFC BCDE(SAC) complex did not display significant DNA binding activity, presumably due to the D(SAC) mutation (Fig. 5C). Surprisingly, when the RFC BCDE(SAC) mutant was titrated into a reaction with the Rh-P/T DNA in the presence of saturating PCNA, a significant amount of DNA binding activity was observed (Fig. 5C). ADP could not substitute for ATPγS to promote this PCNA-dependent DNA binding (Fig. 5C), which may reflect the need for RFC to bind ATP to interact with PCNA. This result demonstrates that PCNA partially rescues the DNA binding deficiency of the RFC D(SAC) mutant.

Removing DNA Interaction Residues in RFC-B, -C, or -D Causes a Deficiency in DNA Binding—The result that RFC D(SAC) is deficient in association with primed template DNA indicates that ATP site C is important in modulating RFC-DNA interaction. Three conserved positively charged residues of RFC-D are predicted to interact with the phosphate backbone of double-stranded DNA that is bound in the central chamber of RFC (Fig. 6A) (20, 31, 32). Previously, we demonstrated that removal of these residues from RFC-D, in combination with removal of the corresponding residues in RFC-B and RFC-C, abolished DNA binding by RFC (36). We used the DNA binding assay described above to test an RFC complex in which only the three RFC-D residues were mutated. The result demonstrates that the RFC D(R101A,R107A,R175A) mutant does not produce a change in fluorescence intensity like wild-type RFC (Fig. 6B), suggesting that removal of these residues is sufficient to break the strong interaction between RFC and the primed template. Interestingly, RFC complexes with the corresponding three mutations in either RFC-B or RFC-C were similarly deficient in DNA binding (Fig. 6B). This disruption of the interac-

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$^3$ G. D. Bowman and J. Kuriyan, unpublished observation.
tion between any one RFC subunit and the phosphate backbone of DNA appears to be sufficient to prevent RFC-DNA interaction.

**The Arginine Fingers of RFC-D and RFC-E Are Most Important for the ATPase Cycle of RFC**—The experiments described above show that the arginine fingers of RFC are not needed for ATP binding or PCNA opening but are needed for DNA binding (i.e., the RFC-D arginine finger). The arginine fingers are also needed for ATP hydrolysis which drives the steps of PCNA loading after formation of the RFC-PCNA-DNA complex, resulting in closure of the clamp and ejection of RFC. Next we examine the effect of individual arginine finger mutations on the steady-state rate of ATP hydrolysis of RFC using a synthetic primed/template in the presence of PCNA. The short linear primed DNA allows PCNA to slide off the DNA after it is loaded, and thus RFC can perform repeated clamp loading events without running out of PCNA and DNA substrate. Wild-type RFC produced a steady-state rate of ~123 ATP hydrolyzed/RFC/min (Fig. 7A). The RFC BCDE(SAC) mutant gave no ATP hydrolysis within the time frame of the assay. Next, we examined the rate of ATP hydrolysis of the single RFC(SAC) mutants. If all four ATP sites contribute equally to the ATPase cycle of RFC, each single RFC(SAC) mutant may be expected to hydrolyze ATP at ~75% the rate of wild-type RFC. However, the results show that two of the RFC(SAC) mutants, RFC D(SAC) and RFC E(SAC), hydrolyze ATP at less than 20% the rate of wild-type RFC. We have shown earlier in this report that the RFC D(SAC) mutant is deficient in DNA binding. If the RFC D(SAC) mutant does not bind DNA, ATP hydrolysis will be compromised since DNA stimulates ATP hydrolysis by RFC (Fig. 7B). However, the ATPase reaction is performed in the presence of sufficient PCNA to enable the RFC D(SAC) mutant to bind to DNA. Therefore the RFC D(SAC) and RFC E(SAC) mutants are likely bound to PCNA and DNA, but the ATP hydrolysis cycle is blocked, preventing clamp loading. One possible explanation for this is that ATP hydrolyze by the remaining ATP sites can fire. The RFC C(SAC) and RFC B(SAC) mutants each hydrolyze ATP at about half the rate of wild-type RFC. Therefore, inability to hydrolyze ATP in sites A or B also hinders hydrolysis in other sites, but the block is less severe than when sites C or D are mutated. These assays were performed in the presence of PCNA and DNA. Addition of either PCNA or DNA alone to RFC also stimulates the rate of ATP hydrolysis by RFC but to a lesser extent than when they are both present with RFC simultaneously.

**FIGURE 5.** The RFC-D arginine finger is required for DNA binding by RFC. The fluorescent primed template contains a 3'-terminal rhodamine moiety attached to the primer strand. Addition of RFC in the presence of ATPγS results in an increase in fluorescence intensity (see scheme at top). A, wild-type RFC and RFC(SAC) mutants were titrated into a reaction containing the fluorescent primed template and the relative fluorescence intensity change (I/I₀) was measured. The Kᵅ values for RFC interaction with the primed template are indicated to the right. B, wild-type RFC was titrated into reactions containing the fluorescent primed template in either the presence of ATPγS (black diamonds) or ADP (red open circles). C, RFC BCDE(SAC) was titrated into reactions containing the fluorescent primed template and ATPγS in the presence (closed circles) or absence (closed diamonds) of a saturating concentration of PCNA (810 nM). RFC BCDE(SAC) was also titrated into the reaction in the presence of ADP and PCNA (crosses).
DNA Triggers ATP Hydrolysis in Site C—At 1 μM DNA, which is saturating in the ATPase assay, ATP hydrolysis by wild-type RFC and all four single RFC(SAC) mutants reaches a maximum, but the RFC D(SAC) mutant was deficient in DNA-stimulated ATP hydrolysis in the presence of PCNA (Fig. 7A and data not shown). This result suggests that DNA may trigger ATP hydrolysis in the unmutated ATP site C of wild-type RFC. To test this possibility we compared the rate of DNA-stimulated ATP hydrolysis of the triple RFC(SAC) mutants. Triple RFC(SAC) mutants contain only one ATP site that is competent for hydrolysis. The RFC BCE(SAC) mutant, which contains only a competent ATP site C, is most strongly stimulated by DNA and in fact displays a rate of ~23 ATP hydrolyzed/RFC/min, about 80% the rate of wild-type RFC (Fig. 7B). This result indicates that DNA triggers ATP hydrolysis in site C and suggests that it may drive hydrolysis in additional ATP sites.

PCNA Triggers ATP Hydrolysis in ATP Site D—PCNA synergistically stimulates ATP hydrolysis by wild-type RFC in the presence of DNA, presumably reflecting repeated cycles of PCNA loading. In contrast, the RFC E(SAC) mutant displayed more steady-state ATP hydrolysis activity in the presence of DNA alone than in the presence of PCNA and DNA (Fig. 7A and data not shown). This PCNA effect is examined more closely in Fig. 8A by titrating PCNA into reactions containing RFC and primed template DNA. The steady-state rate of ATP hydrolysis by wild-type RFC in the presence of primer-template DNA is stimulated by PCNA, while PCNA has the opposite effect on the ATPase activity of the RFC E(SAC) mutant. PCNA inhibition of ATP hydrolysis by the RFC E(SAC) mutant implies that when ATP in site D cannot be hydrolyzed, PCNA blocks the ATPase cycle.

In Fig. 8B we tested the RFC E(SAC) mutant and the other single RFC(SAC) mutants for ATPase activity in the absence and presence of PCNA, without DNA. The RFC E(SAC) mutant is not significantly stimulated by PCNA. This result suggests that association of PCNA with RFC-ATP normally stimulates the hydrolysis of ATP bound to site D. To test this proposal, we examined the triple RFC(SAC) mutants for PCNA-stimulated
The ATP Cycle of RFC

A.

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\begin{align*}
\text{RFC WT-DNA} & \rightarrow \text{RFC WT-PCNA-DNA} \\
\text{ATP} & \rightarrow \text{ADP} + P_i \\
\text{ATP} & \rightarrow \text{ADP} + P_i \\
\text{RFC WT} & \rightarrow \text{PCNA} \rightarrow \text{RFC WT-PCNA-DNA} \\
\end{align*}
\]

B.

**FIGURE 8.** PCNA triggers ATP hydrolysis in ATP site D of RFC. Reactions were performed as in Fig. 7 unless indicated. Error bars indicate one standard deviation of the data set. A, PCNA was titrated into a reaction containing primed DNA and either wild-type RFC or RFC E(SAC); ADP production was analyzed after 2-min incubation. B, ATPase activity of RFC(SAC) single site mutants was analyzed in either the absence (−) or presence (+) of PCNA. C, ATPase activity of RFC(SAC) triple site mutants was analyzed in either the absence (−) or presence (+) of PCNA.

ATPase activity (Fig. 8C). The results show that PCNA has the greatest stimulatory effect on ATP hydrolysis by the RFC BCD(SAC) triple mutant, which only has a competent ATP site D (Fig. 8C). PCNA stimulates the basal rate of the triple RFC BCD(SAC) mutant 10-fold, which is significantly more than the 3–4-fold stimulation by PCNA on wild-type RFC ATP hydrolysis.

**DISCUSSION**

The spiral heteropentameric RFC clamp loader from *S. cerevisiae* has four bipartite ATP sites, each located at subunit-subunit interfaces (see Fig. 1). At each site one subunit contributes a catalytic arginine finger that is contained within a highly conserved SRC motif in RFC box VII. The other subunit contains the P-loop and other ATP binding elements. In this report, conserved arginine fingers are mutated individually and in combination and the resulting ATP site mutant RFC complexes are examined for ability to progress through the different steps in clamp loading. The results reveal individual functions of different ATP sites during the clamp loading cycle.

**PCNA Is Open Prior to ATP Hydrolysis**—In the absence of ATP, neither *E. coli* complex nor eukaryotic RFC bind to their respective clamp or to DNA (28, 48, 59, 60). ATP binding powers a conformational change that enables the clamp loader to bind the clamp and DNA (12, 27, 33, 46, 47). Use of ATPγS promotes ring opening and ring loading onto DNA, but the ring stays open and the clamp loader also remains with the open clamp on DNA. This was demonstrated in the *E. coli* system using a labeled maleimide and a β clamp containing a cysteine residue at a buried position within the clamp interface (27). In the presence of ATPγS, clamp opening is signaled by an increase in reactivity of the buried cysteine residue at the interface. Several lines of evidence exist for RFC holding PCNA open while it is bound to DNA with ATPγS: 1) an electron microscopic reconstruction of a *P. furiosus* RFC-ATPγS-PCNA-
DNA complex shows an open PCNA ring in a right-handed helix (37), 2) A FRET study of yeast RFC indicates that the clamp remains open upon DNA binding (33), similar to the EM study of the \( P. \) furiosus clamp loader, and 3) study of RFC mutants indicates that the ring is open in the RFC-ATP\( /H9253\)S-PCNA-DNA complex and that RFC, not PCNA provides the grip that holds RFC-ATP\( /H9253\)S-PCNA to DNA (36).

The PCNA ring is closed in the RFC-ATP\( /H9253\)S-PCNA structure in which the four arginine fingers of RFC were mutated to glutamine to inhibit nucleotide hydrolysis (20). The reason for this result is not understood at present. However, the studies of the current report show that the RFC with four SRC\( \rightarrow \) SAC mutations (i.e. RFC BCDE(SAC)) is still active in binding and opening PCNA. The RFC BCDE(SAC) mutant is also capable of assembling PCNA onto DNA with ATP\( \gamma S\), forming the RFC-ATP\( \gamma S\)-PCNA-DNA complex (Fig. 5C), which is consistent with the ability of RFC BCDE(SAC) to open PCNA (Fig. 4B, bottom).

The current study of arginine finger mutants of RFC demonstrates that the arginine finger of RFC-D is required for RFC to bind DNA. This ATP site corresponds to site C of \( E. \) coli \( \gamma \) complex (at the interface of two \( \gamma \) subunits), the one site that remains closed in \( \gamma \) complex when it is cocrystallized with ATP\( \gamma S\) (61). Furthermore, the \( \gamma \) complex structure with two bound ATP\( \gamma S\) molecules is in essentially the same conformation as inactive \( \gamma \) complex with no bound nucleotide. Therefore, binding of ATP to site C is presumed to power the conformational change in \( \gamma \) complex required to bind both \( \beta \) and DNA. The ATP site C arginine finger mutation of RFC is the only one that blocks a step powered by ATP binding, and thus one may infer that ATP site C of RFC may carry a similar importance to the ATP-dependent conformation of RFC as ATP site C in \( E. \) coli \( \gamma \) complex. However, structures of the fully ATP bound state of \( \gamma \) complex and of the unliganded state of RFC will be needed to confirm whether this prediction is indeed the case.

The ATP Cycle of RFC—The scheme in Fig. 9 summarizes a model of the ATP hydrolysis cycle of RFC inferred from the findings of this report. ATP binding powers all the steps that
lead to formation of the complex of RFC bound to an open, spiral PCNA clamp (diagram A). To take the next step, binding to DNA, the arginine finger of RFC-D is needed to sense the γ-phosphate of ATP bound to site C. This ATP sensing step brings the RFC-PCNA complex into the proper conformation to bind primed DNA (diagram B). This conformational change could be either subtle, for example by bringing the DNA binding residues of RFC-D into proper alignment for interaction with the phosphate backbone, or could be a more global change, such as altering the spiral pitch of the AAA+ domains of the five subunits of RFC, thus bringing all the subunits into register for DNA interaction. The results presented in Fig. 6B indicate that removal of DNA-interacting residues from either RFC-B, -C, or -D eliminates RFC-DNA contact, suggesting that local changes in RFC-D may be sufficient to modulate DNA binding of the RFC complex.

RFC-PCNA binds to primed DNA through the slot between RFC subunits A and E, and through the open gap in PCNA. Once the DNA and PCNA substrates are properly oriented, the ATP hydrolysis phase may begin. We propose in Fig. 9 that ATP site D may fire first, allowing PCNA to close (diagram C), followed by hydrolysis at sites C, B, and A. We have shown previously that RFC-E, possibly along with RFC-D, opens the PCNA clamp (36). We show here that hydrolysis in ATP site D is specifically stimulated by PCNA, and we propose that hydrolysis leads to PCNA closing around DNA, thereby severing the contact to RFC-D/E (diagram C). ADP formation in the ATP binding pocket of RFC-D (ATP site D) may then act by a local allosteric mechanism to position the RFC-D arginine finger for catalysis of ATP in site C. This wave of cause-and-effect may lead to hydrolysis proceeding around the pentamer to ATP site B and then ATP site A, which is located across the subunit gap from ATP site D. The fact that RFC activity is least affected by mutation of ATP site A, compared with the other ATP sites, is consistent with hydrolysis of this site occurring at the end of the hydrolysis cycle.

Hydrolysis of ATP in site C is sensed by the arginine finger of RFC-D, thus lowering the affinity of RFC for DNA. A decrease in affinity of RFC for DNA is also promoted by the absence of nucleotide in ATP sites C and D as indicated by a study of P-loop mutants of RFC that no longer bind ATP (29). In the model of Fig. 9, ATP hydrolysis continues to site B and then the ATP site of RFC-A is last to fire. RFC-A forms a tight connection to PCNA (20), and hydrolysis in site A may loosen this contact (diagram D). Hence, the ATP hydrolysis cycle of Fig. 9 first causes release of two RFC subunits (D and E) from PCNA, then loosens the interaction of RFC with DNA (site C) followed by severing the remaining contact between RFC-A and PCNA, thus ejecting the clamp loader.

**Comparison with Other Clamp Loaders**—Unlike RFC, E. coli γ complex contains only three ATP sites. These sites correspond to ATP sites B, C, and D of RFC. In γ complex, the arginine finger of ATP site D is contained in 6’ (E subunit), and mutation of this arginine yields a clamp loader complex that has lower affinity for the β clamp (44). Arginine fingers of the other ATP sites cannot be mutated individually since the γ subunits are encoded by the same gene. However, mutation of the arginine fingers in both ATP sites B and C yields a complex that no longer interacts tightly with DNA (44). Thus, there are striking parallels between arginine finger mutants of RFC and γ complex. The arginine finger of RFC in ATP site C is needed for DNA binding and thus has similar behavior to the arginine finger mutation in ATP sites B and C in γ complex. If RFC and γ complex act in a similar fashion, then one may predict that the arginine finger in ATP site C of γ complex, and not the arginine in ATP site B, is responsible for the ATP binding-induced DNA affinity of γ complex. Mutation of the γ complex arginine finger in ATP site D (between the D and E positions), like the same mutation in RFC, prevents ATPase activity in response to the clamp (43). Interestingly, the RFC-D and RFC-E subunits of RFC are needed to open the PCNA clamp (36). However, in a significant departure between the activities of the eukaryotic RFC and γ complex, the δ wrench (A subunit) of γ complex opens the β clamp, while RFC-A lacks this activity (36). It is interesting to note that the proposed order of ATP hydrolysis in γ complex (43) is also opposite to that proposed here for RFC.

The RFC of Archaeoglobus fulgidus is similar to E. coli γ complex and eukaryotic RFC in the ability to load a clamp onto DNA prior to ATP hydrolysis, and in all cases the clamp loader remains on DNA with the clamp. One may presume that the clamp remains open from the work in the E. coli system and from the electron micrographic reconstruction of the RFC-ATPγS-PCNA-DNA complex from the archaeon P. furiosus (37). Archael RFC complexes consist of two different subunits: RFC-s (s for small) and RFC-I (I for large), in a 4:1 ratio, respectively. Studies on A. fulgidus RFC suggest that four ATP molecules bind to archaeal RFC and are hydrolyzed during a full clamp loading cycle (49). A. fulgidus RFC with arginine finger mutations in the four RFC-s subunits still binds ATP but is no longer functional in formation of the RFC-PCNA-DNA ternary complex (62), suggesting that a step prior to hydrolysis is disrupted by this mutation. This step correlates with the finding here that the arginine finger of RFC-D is needed to sense ATP binding in site C for RFC to bind DNA.

Clamp loader subunit sequences and clamp loader structures are similar in all three domains of life: bacteria, archaea, and eukaryotes (20, 21, 34, 37, 63–66). ATP sites C and D in RFC, which are critical for efficient PCNA loading, also are present in bacterial and archaeal clamp loaders. These two ATP sites are also present in the alternate RFC clamp loaders in which the A subunit (RFC1) exchanges with another protein (e.g. Rad24 (Rad17 in humans), Ctf18, or Elg1). These “alternate” RFC complexes act in other cellular processes such as DNA damage repair and sister chromatid cohesion (15). For example, the Rad24 subunit substitutes for RFC-A to form Rad24-RFC which loads the 9-1-1 heterotrimer clamp onto DNA (67, 68). The Ctf18-RFC acts in sister chromatid cohesion and is efficient in unloading PCNA, leading to the suggestion that it may serve in this capacity when replication forks deal with cohesins (69–71). Since ATP sites C and D are present in all alternate clamp loaders, these two sites may be expected to perform similar functions to those shown here for the replicative RFC complex.
