The eukaryotic replication factor C (RFC) clamp loader is an AAA+ spiral-shaped heteropentamer that opens and closes the circular proliferating cell nuclear antigen (PCNA) clamp processivity factor on DNA. In this study, we examined the roles of individual RFC subunits in opening the PCNA clamp. Interestingly, Rfc1, which occupies the position analogous to the β clamp-opening subunit in the *Escherichia coli* clamp loader, is not required to open PCNA. The Rfc5 subunit is required to open PCNA. Consistent with this result, Rfc2:3:4:5 and Rfc2:5 subassemblies are capable of opening and unloading PCNA from circular DNA. Rfc5 is positioned opposite the PCNA interface from Rfc1, and therefore, its action with Rfc2 in opening PCNA indicates that PCNA is opened from the opposite side of the interface that the *E. coli* δ wrench acts upon. This marks a significant departure in the mechanism of eukaryotic and prokaryotic clamp loaders. Interestingly, the Rad-RFC DNA damage checkpoint clamp loader unloads PCNA clamps from DNA. We propose that Rad-RFC may clear PCNA from DNA to facilitate shutdown of replication in the face of DNA damage.

The proliferating cell nuclear antigen (PCNA) DNA sliding clamp is a ring-shaped homotrimer that is opened and closed around DNA by the replication factor C (RFC) clamp loader (1–4). The PCNA clamp then binds numerous different proteins, including the cellular replicases DNA polymerase δ and DNA polymerase ε (5). The RFC clamp loader was initially identified as a factor required for SV40 DNA replication *in vitro* (2). Subsequent characterization of RFC showed it to consist of five different subunits in both yeast and human cells (6). The five subunits are homologous to one another and are members of the AAA+ family of ATPases (7). The structures of yeast RFC and the *Escherichia coli* clamp loader γ complex reveal that the subunits are arranged in a similar spiral fashion (8, 9). To avoid confusion over the different nomenclatures used for the different systems, the positions of the various clamp loader subunits are designated A–E as listed in Table 1. The position of each subunit in the complex is indicated in parentheses with a letter when comparisons are made between systems. The yeast Rfc1(A) subunit (human p140(A)) is sometimes referred to as the large subunit, as it contains both N- and C-terminal extensions past its region of homology with the four small subunits. The other four RFC subunits, referred to as the small subunits, are in the 36–40 kDa range: yeast Rfc2(D) (human p37), yeast Rfc3(C) (human p36), yeast Rfc4(B) (human p40), and yeast Rfc5(E) (human p38). The N-terminal region of Rfc1, up to the region of homology to the other RFC subunits, can be deleted in both yeast and human RFC without decreasing RFC clamp loader function with PCNA (10, 11). Alternative clamp loaders exist in which Rfc1 is replaced by another protein. An example of one such alternative clamp loader is the Rad-RFC DNA damage checkpoint clamp loader, in which Rad24(A) (Rad17 in humans) replaces Rfc1(A) and loads the 911 heterotrimer clamp onto DNA (12).

RFC shares striking structural and functional similarity with the *E. coli* clamp loader γ complex (1, 8, 9, 13, 14). Both RFC and the *E. coli* γ complex require the binding of ATP or ATPγS to induce a conformational change that allows interaction with the clamp and subsequent ring opening (15–19). The nature of this conformational change is unclear, but ATP binding is sufficient, and hydrolysis is not needed. The γ complex consists of five clamp loader subunits, three γ, one δ, and one ε, each of which are composed of three domains (9). Two small subunits of the γ complex, γ and δ, are not required for the clamp-loading operation. The first two N-terminal domains of the γ, δ, and ε subunits consist of the AAA+ region of homology. The crystal structure reveals that the C-terminal domain of each subunit mediates oligomerization into a spiral heteropentamer and that a gap exists between the AAA+ regions of the δ(A) and δ(E) subunits (9). The δ subunit binds β tightly and can open the β clamp by itself (20). The crystal structure of the δ “wrench” bound to β reveals that β binds the N-terminal AAA+ domain of the δ subunit (21). Superposition of δβ onto γ,δε indicates that β likely docks onto the AAA+ domains of all five clamp loader subunits (9).

The structure of RFC in complex with PCNA and ATPγS shows that the RFC subunits have a similar domain arrangement and circular architecture to the γ complex (8). RFC retains the pronounced gap between the AAA+ domains of the Rfc1(A) and Rfc5(E) subunits, which correspond spatially to the δ(A) and δ(E) subunits in the γ complex. This gap serves the purpose of allowing DNA to enter the central chamber in the clamp loader and thereby positions DNA into the opened clamp docked underneath the clamp loader. The C-terminal extension of Rfc1 is positioned within a portion of this gap.

Rfc1(A) is located in the position analogous to the δ(A) wrench in γ,δε. However, the RFC-PCNA-ATPγS structure reveals a closed PCNA ring despite interaction with Rfc1. This closed conformation of PCNA may possibly be due to specific mutations that were made in RFC to prevent nucleotide hydrolysis during crystal growth. The Rfc3 and Rfc4 subunits also bind PCNA. Due to the spiral structure of RFC in complex with a flat planar PCNA ring, each adjacent RFC subunit is poised further away from the PCNA ring. Thus, in going around the RFC pentamer, Rfc1(A) forms the most extensive contact with PCNA;
Mechanism of PCNA Clamp Opening

The five subunits of the clamp loader are arranged in a spiral, with each position designated by a letter.

| CLAMP LOADER | \( \text{A} \) | \( \text{B} \) | \( \text{C} \) | \( \text{D} \) | \( \text{E} \) |
|---------------|---|---|---|---|---|
| \( \text{S. cerevisiae RFC} \) | RFC1 | RFC4 | RFC3 | RFC2 | RFC5 |
| \( \text{S. cerevisiae Rad-RFC} \) | Rad24 | RFC4 | RFC3 | RFC2 | RFC5 |
| \( \text{human RFC} \) | p140 | p50 | p36 | p37 | p38 |
| \( \text{E. coli } \gamma \text{ complex} \) | \( \delta \) | \( \gamma_1 \) | \( \gamma_2 \) | \( \gamma_1 \) | \( \delta' \) |

Rfc4(E) is positioned above an interface and contacts PCNA in a minimal fashion; then Rfc3(C) binds the second protomer of PCNA, but buries less surface area than the Rfc1-PCNA contact; and finally, Rfc2(D) and Rfc5(E) are suspended above PCNA and do not bind the ring at all. Like Rfc4, Rfc2 is positioned above a PCNA interface. Rfc5 is positioned above the remaining hydrophobic site in the third PCNA protomer.

In this study, we examined RFC subunits for the ability to open PCNA, which was observed experimentally by the unloading of PCNA from circular DNA. This work demonstrates that Rfc1 is not required for PCNA opening. In fact, the Rfc2-3-4-5 subcomplex of the four small RFC subunits can open PCNA clamps and clear them from DNA. This study further demonstrates that Rfc2 and Rfc5 bind directly to PCNA and that Rfc5 is required for PCNA opening by RFC. In fact, an Rfc2-5 subcomplex can open and unload PCNA from DNA. Rfc5(E) is positioned opposite the PCNA interface from Rfc1(A), and therefore, its action with Rfc2 in opening PCNA indicates that the PCNA clamp is opened from the opposite side of the interface that the \( \delta \) wrench acts upon. These internal workings mark a significant departure in the underlying mechanism of clamp opening by eukaryotic and prokaryotic clamp openers.

EXPERIMENTAL PROCEDURES

Materials

Radioactive nucleotides were purchased from PerkinElmer Life Sciences. DNA modification enzymes were from New England Biolabs, and DNA oligonucleotides were from Integrated DNA Technologies. RFC concentrations were determined using the Bio-Rad protein stain and bovine serum albumin as a standard. PCNA concentration was determined by absorbance at 280 nm using a molar extinction coefficient of 6420 \( \text{M}^{-1} \text{cm}^{-1} \). Buffer A contained 30 mM HEPES (pH 7.5), 10% (v/v) glycerol, 0.5 mM EDTA (pH 7.5), 1 mM dithiothreitol (DTT), and 0.04% Bio-Lyte 3/10 (Bio-Rad). Buffer B was composed of 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.5 mM EDTA, and 10% glycerol. Buffer C was composed of 20 mM Tris-Cl (pH 7.9), 10% sucrose, 500 mM NaCl, and 4% glycerol. Buffer D contained of 20 mM Tris-HCl (pH 7.9), 0.04% Bio-Lyte 3/10, and 4% glycerol. Buffer E contained 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, and 4% glycerol. Surface plasmon resonance buffer was composed of 10 mM HEPES (pH 7.5), 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P2. Clamp loading buffer contained 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 40 \( \mu \text{g/mL} \) bovine serum albumin, and 5% glycerol. Gel filtration buffer was composed of 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.5 mM EDTA, 100 \( \mu \text{g/mL} \) bovine serum albumin, 8 mM MgCl\(_2\), 4% glycerol, and 150 mM NaCl.

DNA Substrates

Singly nicked plBluescript was prepared as described (22) using M13 gplI endonuclease and supercoiled plBluescript SK\(^+\) plasmid (Stratagene, La Jolla, CA) until 50% of the replicative form I DNA was converted to replicative form II DNA and then was purified by phenol extraction. Singly primed single-stranded M13 DNA was prepared by annealing the synthetic DNA primer 5’-cga gct gtt aac acg agc gat gcc aag ctt gca tgc ctg cag gtc gac tct aga gga ggg tag cat atg ctt ccc-3’ to purified single-stranded circular M13EBNA1 DNA (refer to Ref. 23 for both a description of the template and the procedure for annealing). The 3‘-end of this primer contains an 18-nucleotide EBNA1-binding sequence that hybridizes to the complementary sequence on the M13EBNA1 template.

Cloning of Mutant RFC Genes

The Saccharomyces cerevisiae RFC genes were cloned into the pET11a (Novagen), pLANT2/RIL (24), or pCDfDuet (Novagen) vector. The plasmids containing single genes include pET11a-RFC1, pET11a-RFC2, pET11a-RFC3, pET11a-RFC4, and pET11a-RFC5. The expression plasmids containing two or more genes include pET11a-RFC3+4, pLANT2/RIL-RFC1+5, and pET11a-RFC2+3+4.

RFC DNA-binding Mutant

Mutations were introduced into the RFC2, RFC3, and RFC4 genes using the QuikChange method (Stratagene). Arg\(^{107}\), Arg\(^{175}\) of RFC2 (RFC2(DNA-bind mut)); Arg\(^{24}\), Arg\(^{25}\), and Lys\(^{156}\) of RFC3 (RFC3(DNA-bind mut)); and Arg\(^{84}\), Arg\(^{90}\), and Lys\(^{149}\) of RFC4 (RFC4(DNA-bind mut)) were all mutated to alanine.

RFC2(DNA-bind mut) was generated in pET11a-RFC2 using the following oligonucleotides: 5‘-gag tgt aag gct gtt ctc gta gaa cag ggt aat gaa aat ttg gct gaa cag gcc ctg cgc ctc cgg 3’ (to introduce Rfc2(R101A/R107A)) and 5‘-gcc gaa aag gta aat tct gcg aag gct gtt ctc ggc cta gct ggc ctc cgg 3’ (to introduce Rfc2(R175A)). RFC3(DNA-bind mut) was generated in pET11a-RFC3+4 using the following primers: 5‘-gag tgt aag gct gtt ctc gta gaa cag ggt aat gaa aat ttg gct gaa cag gcc ctg cgc ctc cgg 3’ (to introduce Rfc3(R88A/R94A)) and 5‘-gta gta gcc gtt ctc gct gaa cag gta aat tct gcg aag gct gtt ctc gta gaa cag ggt aat gaa aat ttg gct gaa cag gcc ctg cgc ctc cgg 3’ (to introduce Rfc3(K152A)). RFC4(DNA-bind mut) was generated in pET11a-RFC4 using the following primers: 5‘-gag tgt aag gct gtt ctc gta gaa cag ggt aat gaa aat ttg gct gaa cag gcc ctg cgc ctc cgg 3’ (to introduce Rfc4(R84A/R90A)) and 5‘-ct gta gtc ggc gca gtc ggt gtc ggc gca gtc ggt gtc ggc gca gtt ctc gct gct ggc 3’ (to introduce Rfc4(K49A)). The open reading frames of the mutated genes were confirmed by DNA sequencing.

To construct pET-RFC(2DNA-bind mut+3DNA-bind mut+4DNA-bind mut), plasmids pET11a-RFC4(DNA-bind mut) and pET11a-
Mechanism of PCNA Clamp Opening

RFC(DNA-bind mut +4) were digested with SacI/AflII. The fragment containing RFC(3DNA-bind mut +4) was ligated into the fragment containing pET11a-RFC3(DNA-bind mut) to yield pET11a-RFC(2DNA-bind mut +3DNA-bind mut +4DNA-bind mut). In addition, pET11a-RFC2(DNA-bind mut) was digested with BglII/HindIII and blunt-filled with Klenow fragment; the small fragment was gel-purified and ligated into pET-RFC(3SAC+4SAC) (which was cut with SphI, blunted with Klenow fragment, and treated with alkaline phosphatase; the large fragment was gel purified). The screen for orientation showed that RFC2 was facing in the opposite orientation from RFC3 and RFC4. Finally, both pET-RFC(3DNA-bind mut +4DNA-bind mut) and pET-RFC(2DNA-bind mut +3SAC +4SAC) were digested with MluI/SgrAI; the large fragment from the former and the small fragment from the latter were ligated together to form pET-RFC(2DNA-bind mut +3DNA-bind mut +4DNA-bind mut).

Truncation Mutations in the RFC1 and RFC5 Genes

RFC(ΔRfc1 AAA +)–Rfc1 lacking the N-terminal ligase region has four remaining domains. The AAA+ region of homology is contained in the first two domains (domains 1 and 2), and domain 3 interacts with the other RFC subunits to form the pentameric collar; domain 4 is a C-terminal extension that is positioned within the gap between Rfc1 and Rfc5 in the crystal structure (9). Domain 3 of Rfc1 (Leu499–Thr567) was isolated from pET11a-RFC1 by PCR using the following primers: 5′-ggg aaa aga ata tgc taa cac tgt tgg ccc-3′ and 5′-ccg gag cat cct gat tgg aaa ttg att ctt ccc gcc atg tgg ccg gca act ttt gat gc-3′. This fragment, lacking domains 1, 2, and 4 (i.e. ΔRfc1 AAA+), was cloned into the Ndel and BamHI sites of the pCDFduet vector to make pCDFduet-ΔRFC1 AAA+. The open reading frame was confirmed by DNA sequencing.

RFC(ΔRfc5 AAA +)–Rfc5(domain 3), which interacts with the other subunits to form the collar of pentameric contacts, corresponds to Pro257–Asp354. The vector for expression of this domain was generated using the same protocol as described above, except that the template DNA used was pET-RFC5. The PCR primers were 5′-ccc tat aca tat gcc ctc ctc atg gat gcc ctc ctc aac gaa tca aca gag-3′ and 5′-gct cgg atc ctc atg gtc gtc act ac-3′. This fragment, lacking domains 1, 2, and 4 (i.e. ΔRfc5 AAA+), was cloned into the Ndel and BamHI sites of the pCDFduet vector to make pCDFduet-ΔRFC5 AAA+.

Overexpression of S. cerevisiae RFC Proteins in E. coli

Transformation reactions utilizing pET-, pLANT/RIL-, and pCDFduet-derived RFC subunit expression plasmids were performed using the BL21(DE3) Star strain of E. coli competent cells (Invitrogen). Transformants were selected on LB plates containing ampicillin (100 mg/ml), kanamycin (50 μg/ml), and streptomycin (50 mg/ml). Fresh transformants were grown in 12–24 liters of Terrific broth containing appropriate antibiotics at 30 °C until cell growth reached an OD600 of 0.6. Cultures were then brought to 15 °C by chilling on ice before addition of 1 mM isopropyl β-D-thiogalactopyranoside, followed by incubation at 15 °C for ∼18 h. Cells were harvested by centrifugation.

Purification of RFC Complexes and Individual Subunits

For purification of the RFC DNA-binding mutant, the five-subunit RFC containing DNA-binding mutations in Rfc2–4 was expressed by cotransformation of pLANT2/RIL-RFCS, pET11a-RFC2+3+4, and pET-RFC5(AAA+). Purification of the RFC mutant followed the same procedure as described for the RFC DNA-binding mutant, except that a 100-ml Q-Sepharose Fast Flow column (Amersham Biosciences) equilibrated with Buffer B containing 90 mM NaCl and eluted with a 200-ml gradient of 90–500 mM NaCl in Buffer B. Fractions containing the RFC complex (which eluted at ∼300 mM NaCl) were stored at −80 °C. The final yield was ∼40 mg of purified RFC(ΔRfc5 AAA+) per liter of culture.

Purification of RFC Complexes Containing Either Truncated Rfc1 or Rfc5

RFC(ΔRfc1 AAA +) containing truncated Rfc1 (domain 3 only) was expressed by cotransformation of pLANT2/RIL-RFCS, pET11a-RFC2+3+4, and pCDFduet-ΔRFC1 AAA+. Purification of the RFC mutant followed the same procedure as described above, except that a 100-ml Q-Sepharose Fast Flow column was used with a 1-liter gradient of 90–500 mM NaCl in Buffer B. The final yield was ∼49 mg of purified RFC(ΔRfc1 AAA+) per liter of culture.

Purification of RFC5(domain 1)

HK-pET-RFC5(domain 1) was transformed and expressed in BL21(DE3) Star competent cells. The cell pellet harvested from 12 liters of culture was resuspended in 180 ml of Buffer C. After lysis using a French press and clarification by centrifugation, the supernatant was diluted with Buffer D to a conductivity equal to 150 mM NaCl. At this conductivity, the RFC5(domain 1) protein flowed through both an SP-Sepharose and a Q-Sepharose Fast Flow column. The protein was then precipitated using 70% ammonium sulfate and resuspended in 60 ml of Buffer E. The protein was dialyzed against Buffer E containing 500 mM NaCl and then applied to a 10-ml nickel-charged chelating Sepharose column and eluted using a 100-ml gradient of 5–1000 mM imidazole in Buffer E. Column fractions were analyzed by 10% SDS-polyacrylamide gels stained with Coomassie Blue. Fractions containing RFC5(domain 1) were pooled and dialyzed against Buffer E. The final yield was ∼10 mg of purified RFC5(domain 1) per liter of culture.

Purification of Wild-type RFC, Subcomplexes, and Individual Subunits

Wild-type RFC with either full-length Rfc1 or Rfc1 lacking the N-terminal ligase domain (referred to here as RFC1ΔNL) was purified...
as described (25). Rfc2, Rfc3-4, and Rfc2-5 were also purified as described (25). The four-subunit Rfc2-3-4-5 complex was reconstituted by mixing equimolar Rfc2-5 and Rfc3-4, followed by incubation at 16 °C for 10 min.

**Purification of Rad-RFC**

The alternative clamp loader Rad-RFC was expressed by cotransformation of pLANT2/RIL-Rad24+RFC5 and pET11a-RFC2-3-4. Cells harvested from 12 liters of culture were resuspended in 175 ml of Buffer A containing 800 mM NaCl and then lysed using a French press at 22,000 p.s.i. The cell lysate was clarified by centrifugation and diluted with Buffer A until the conductivity was 200 microsiemens/cm and the volume was 500 ml. The lysate was then applied to a 125-ml SP-Sepharose Fast Flow column equilibrated with Buffer A containing 150 mM NaCl. The column was eluted with a 1-liter gradient of 150–600 mM NaCl in Buffer A. Fractions containing the Rad-RFC complex were stored at 80 °C. The final yield was ~120 mg of purified Rad-RFC/12 liters of culture.

**Surface Plasmon Resonance**

Rfc5(domain 1) was immobilized on a CM5 sensor chip using carbodiimide chemistry in 10 mM sodium acetate (pH 4.5) to yield a final value of ~4000 response units of immobilized Rfc5(domain 1). 1 μM protein kinase-tagged PCNA in surface plasmon resonance buffer containing 8 mM MgCl₂ was passed over immobilized Rfc5(domain 1) at a flow rate of 6 μl/min for 5 min, after which buffer lacking protein was injected over
**Mechanism of PCNA Clamp Opening**

**Gel Filtration Analysis**

A six-residue N-terminal kinase recognition motif was cloned into *S. cerevisiae* PCNA, allowing it to be radiolabeled with $[^{32}P]-32P$-ATP using the recombinant catalytic subunit of cAMP-dependent protein kinase (26, 27). $[^{32}P]$-PCNA (~20–60 cpm/fmol) was first loaded onto either singly nicked plasmid or singly primed single-stranded M13 DNA (as indicated in the figure legends). Reactions contained either 4 pmol of gpII-nicked pBluescript or 2.8 pmol of singly primed single-stranded M13 DNA (coated with ~757 pmol of *E. coli* single-stranded DNA-binding protein as tetramer), 10 pmol of $[^{32}P]$-PCNA, and 2 pmol of RFC1 in 200 l of clamp loading buffer containing 2 mM ATP and 8 mM MgCl$_2$. After 20 min at 30 °C, the $[^{32}P]$-PCNA-DNA complex was purified from free $[^{32}P]$-PCNA by gel filtration on 5-ml Bio-Gel A-1.5m columns (Bio-Rad) equilibrated with gel filtration buffer containing 150 mM NaCl. 180-l fractions were collected. Excluded peak fractions, 13–17, were combined and then divided into four tubes (210 l each). Each tube was incubated for 5 min at 30 °C with 2 mM ATP in the presence or absence of wild-type or mutant RFC (exact amounts are given in the figure legends). The linear range of the assay extends to 70% of clamps unloaded. Therefore, initial experiments were performed to determine the concentration range of wild-type and mutant RFC and subcomplexes that fall within the linear range for the experiments presented. Reactions were then examined for $[^{32}P]$-PCNA clamp loading by gel filtration on a second 5-ml Bio-Gel A-1.5m column equilibrated with gel filtration buffer containing 150 mM NaCl, and 180-l fractions were collected. Aliquots (150 l) were analyzed for radioactivity by liquid scintillation counting. The molar amount of $[^{32}P]$-PCNA in each fraction was calculated from the known specific activity of radioactive PCNA.

**Fluorescent DNA Binding Assays**

RFC binding to DNA was measured using a fluorescent primed DNA template. The fluorescent 30-mer oligonucleotide primer contained a Rhodamine Red-X fluorophore group (Integrated DNA Technologies) linked to the 3'-OH by a six-carbon spacer. The primed DNA template was prepared by annealing 1.0 nmol of 3'-Rhodamine Red-X-conjugated 30-mer with 1.2 nmol of unlabeled 66-mer in 100 l of 5 mM Tris-HCl, 150 mM NaCl, and 15 mM sodium citrate (final pH 8.5) as described above for unlabeled templates. RFC complexes were titrated from 0 to 120 nM into 60-l reactions containing 20 mM Tris (pH 7.5), 125 mM NaCl, 8 mM MgCl$_2$, 2 mM DTT, 0.04 mg/ml bovine serum albumin, 0.5 mM EDTA, 100 mM ATPγS, and 10 mM rhodamine-labeled primertemplate. The fluorophore was excited at 570 nm, and emission was monitored over 580–680 nm. The relative intensity of the peak at 588 nm was plotted versus RFC concentration. The apparent $K_d$ for wild-type RFC binding to DNA was determined using the model A + B $\rightleftharpoons$ AB and the curve-fitting software KaleidaGraph (Synergy Software).

**RESULTS**

**Clamp Unloading as a Means for Measuring PCNA Opening**—One of the primary steps in RFC assembly of PCNA on DNA is the opening of the clamp. Clamp loaders (both prokaryotic and eukaryotic) can also unload clamps from DNA (3, 28–30). ATP is required in both directions (28), and therefore, the forward (loading) and reverse (unloading) reactions do not constitute a true equilibrium. Opening of the clamp is required for both reactions, but loading clamps onto DNA has the addi-
unallooding by wild-type RFC in a recent study (33). Presumably, wild-type _S. cerevisiae_ RFC may also be expected to unload PCNA. Fig. 1 illustrates an unloading reaction using a version of yeast RFC that we used throughout this study. This particular RFC contains an Rfc1 subunit that lacks the N-terminal 282 amino acids, which we refer to as RFC_{1,ΔN}. The N-terminal region of Rfc1 binds DNA nonspecifically, and removal of this region has no observable replication phenotype and results in a more active RFC clamp loader relative to the wild type (10, 11, 31, 32).

The experiment in Fig. 1 compares the unloading activity of RFC_{1,ΔN} with that of wild-type RFC. The result shows that RFC_{1,ΔN} removed PCNA clamps from circular duplex DNA (Fig. 1A), but wild-type RFC, containing full-length Rfc1, was much less efficient in this unloading reaction (Fig. 1B). This result is consistent with the inability to observe unloding by wild-type RFC in a recent study (33). Presumably, wild-type RFC is a poor clamp unloader compared with its activity as a clamp loader, and the reaction lies far in the direction of clamps being assembled onto DNA, even at very low concentrations of free PCNA. The previous study did not detect unloading by RFC_{1,ΔN}, but this discrepancy may be explained by the higher amounts of protein used here.

**Rfc1 Is Not Required for PCNA Opening**—The touch points between PCNA and RFC are located within the N-terminal ATPase domains of each subunit contained within the AAA+ region of homology (8). The AAA+ domains are the first two (i.e. N-terminal) of the three domains that compose the small RFC subunits, Rfc2–5. The third domain (i.e. C-terminal) mediates the major pentameric contacts that form a “collar” that holds the subunits together. Rfc1 contains these three domains as well, but also contains N- and C-terminal extensions relative to the small subunits. If Rfc1 acts in a fashion analogous to the δ wrench in the γ complex, the Rfc1 AAA+ region, which contacts the PCNA clamp, should be required for clamp opening. Hence, we expressed an RFC mutant in which the N and C termini of Rfc1 were pared down to only the “collar domain,” which mediates oligomerization with the other subunits. We refer to this RFC mutant as RFC(ΔRfc1 AAA+); it behaved similarly to wild-type RFC and RFC_{1,ΔN} during purification. Although the RFC(ΔRfc1 AAA+) mutant was inactive in loading PCNA onto DNA (data not shown), analysis of 32P-PCNA removal from DNA showed that this RFC mutant retained clamp unloading activity and was thus still capable of opening PCNA (Fig. 2A). The amount of PCNA released from DNA by RFC(ΔRfc1 AAA+) was similar to that released by RFC_{1,ΔN} (compare Figs. 1A and 2A). This result suggests that interaction between PCNA and the AAA+ domain of Rfc1, which includes the PCNA-binding region, is not required for PCNA clamp opening.

**Rfc5 Is Required for PCNA Opening**—We also expressed an RFC mutant missing the AAA+ region of Rfc5, but containing the C-terminal collar domain of Rfc5, as well as full-length Rfc2–4 and RFC_{1,ΔN}.

![Mechanism of PCNA Clamp Opening](image-url)

**FIGURE 3. RFC subcomplexes containing Rfc5 can open PCNA to unload the clamp.** Clamp unloading reactions were performed using singly primed M13mp18 single strand DNA coated with _E. coli_ single-stranded DNA-binding protein and Rfc2–3-4-5 (A), Rfc2–5 (B), or Rfc3–4 (C). Reactions were performed as described under “Experimental Procedures.” The red circles are controls in which the 32P-PCNA-DNA complex was treated with buffer only. RFC subcomplexes were titrated in PCNA unloading assays, and the amount of 32P-PCNA unloaded from DNA was quantitated (D).

This RFC mutant is referred to here as RFC(ΔRfc5 AAA+). In contrast to RFC(ΔRfc1 AAA+), the RFC(ΔRfc5 AAA+) mutant could not unload PCNA (Fig. 2B), implying that contact between Rfc5 and PCNA is needed to open the PCNA clamp.
Mechanism of PCNA Clamp Opening

To further investigate the role of Rfc5 in PCNA opening, we studied subcomplexes of RFC. Because Rfc1-PCNA interaction is not necessary for clamp opening, one may predict that an Rfc2/Rfc5 subcomplex, lacking Rfc1 entirely, will still open and unload PCNA from DNA. Fig. 3A demonstrates that the Rfc2/Rfc5 subcomplex was able to unload PCNA from DNA. This result is consistent with an observation in the human system indicating that an RFC subassembly analogous to Rfc2/Rfc5 can unload PCNA (30).

Next, we purified the Rfc3/Rfc4 and Rfc2/Rfc5 subcomplexes. Results using these heterodimers revealed that unloading activity lies in the Rfc2/Rfc5 complex; Rfc3/Rfc4 lacks PCNA unloading activity (Fig. 3, B and C). Experiments using the individual Rfc2 and Rfc4 subunits showed no clamp unloading activity, indicating that PCNA opening requires Rfc5 (either alone or with Rfc2) (data not shown). We were unable to obtain the isolated Rfc5 or Rfc3 subunit in a soluble form in this study.

Next, we examined the efficiency of these complexes in PCNA unloading relative to RFC1AAA (Fig. 3D). The results demonstrate that Rfc2-/Rfc5 was ~100-fold less efficient than RFC1AAA. The lower efficiency of Rfc2-/Rfc5 compared with RFC(ΔRfc1 AAA +) suggests that disruption of the circular collar affects the efficiency of the clamp unloading activity. The unloading activity of Rfc2/Rfc5 was comparable with that of Rfc2-/Rfc5. These results suggest that Rfc2/Rfc5 is primarily responsible for the PCNA clamp-opening function of RFC.

Rfc2 and Rfc5 Bind PCNA—The above results demonstrate that Rfc2/Rfc5 unloads PCNA and therefore suggest that one or both of these subunits interact with PCNA. The RFC-PCNA-ATPγS structure shows no contact between Rfc2 and Rfc5 with the closed PCNA ring (8). However, molecular simulations indicate that the PCNA ring opens out of plane to form a right-handed spiral (34). The bottom diameter of RFC matches the dimensions of the PCNA ring, and thus, it seems likely that all of the RFC subunits contact PCNA in the open spiral form. Indeed, an electron microscopic reconstruction study of an archaeal RFC-PCNA-ATPγS complex indicated that the open PCNA ring interacts extensively with RFC (35). Hence, we examined Rfc2 and Rfc5 for direct contact with PCNA.

Full-length Rfc2 can be purified individually as recombinant protein expressed in E. coli (25). However, we have not been able to obtain soluble Rfc5 when expressed without other subunits. We have also tried expressing Rfc5 as a fusion with either galactose- or maltose-binding protein at the N or C terminus, but the resulting fusions were completely insoluble. However, we were successful in expressing and purifying the N-terminal AAA domain of Rfc5.

Interaction of Rfc2 and Rfc5(domain 1) with PCNA was studied by surface plasmon resonance (Fig. 4). Either Rfc2 or Rfc5(domain 1) was immobilized on the sensor chip, and a solution of PCNA was passed over the top. The results show a clear interaction of both Rfc2 and Rfc5(domain 1) with PCNA. Similar analyses in which PCNA was...
immobilized, and Rfc2 or Rfc5(domain 1) was passed over it also yielded an interaction between them (data not shown). The $K_d$ values calculated from the data of Fig. 4 are $\sim$480 nM for Rfc2-PCNA and $\sim$50 $\mu M$ for Rfc5(domain 1)-PCNA. These values are 10- and 1000-fold weaker, respectively, than the $K_d$ value reported for RFC-PCNA determined by surface plasmon resonance in an earlier study (10). Hence, the full strength of interaction between RFC and PCNA likely requires all or most of the subunits.

DNA Binds within the Central Chamber of RFC—The RFC-PCNA-ATP\gammaS structure implies that DNA binds within the central chamber of RFC, defined by the spiral circular arrangement of the AAA+ domains of the RFC subunits (Fig. 5A) (1, 8). The Rfc1–4 subunits contain several conserved positively charged and polar side chains that are oriented toward the central chamber and may function to hold DNA within the RFC-PCNA complex (8). Several of these side chains are conserved in both prokaryotic and eukaryotic clamp loader subunits. Experimental evidence that clamp loaders bind DNA within this central chamber derives from studies of the E. coli $\gamma$ complex showing that mutation of these conserved residues reduces DNA binding (36).

To test these residues for DNA binding in the central chamber of RFC, we mutated three conserved positively charged residues in each of the Rfc2–4 subunits and purified the resulting RFC mutant (referred to here as the RFC DNA-binding mutant). These residues are near the DNA modeled into the center of RFC (Fig. 5A). Next, we developed a fluorescent DNA binding assay using two short synthetic oligonucleotides that hybridize to form a primed template with a rhodamine moiety on the 3'-nucleotide of the primer to report RFC binding. Titration of RFC into the fluorescent primed template in the presence of ATP\gammaS resulted in a change in intensity with an observed $K_d$ of 29.7 nM (Fig. 5B). This value compares favorably with the previously observed $K_d$ of 15 nM for yeast RFC binding to a primed site with ATP\gammaS (no PCNA) determined using a Biacore system (10).

Analysis of the RFC DNA-binding mutant in the fluorescent DNA binding assay demonstrated vastly reduced DNA binding (Fig. 5B). Assuming that full binding produces a similar maximal intensity change as RFC, the $K_d$ value for interaction of the RFC mutant with DNA is $\sim 1 \mu M$. It remains possible that one or more of the amino acid replacements may have altered the proper folding of the RFC DNA-binding mutant. However, the ability of the RFC DNA-binding mutant to bind and unload PCNA from DNA indicates that the RFC mutant is properly folded (described below).

ATP Hydrolysis Is Not Required to Open PCNA for Clamp Unloading—In Fig. 6, we examined the ability of the RFC DNA-binding mutant to function with PCNA in clamp loading and unloading assays. As expected, the RFC mutant was unable to load PCNA onto DNA (Fig. 6A). However, the RFC mutant inhibited PCNA loading when mixed with wild-type RFC (Fig. 6A). This observation indicates that the RFC DNA-binding mutant is still able to bind PCNA, sequestering it away from active RFC.

Next, we examined the RFC DNA-binding mutant for ability to unload $32$P-PCNA from DNA (Fig. 6B, right panel). Surprisingly, the RFC DNA-binding mutant could still unload clamps from DNA (Fig. 6B). It is interesting to note that the PCNA unloading activity of the RFC DNA-binding mutant was observed even when ATP\gammaS was substituted for ATP. With RFC, ATP\gammaS promoted ring opening and PCNA loading, but RFC stayed with PCNA on DNA; therefore, clamp unloading was not observed (Fig. 6B, left panel). This is presumably due to the tight interaction of the RFC-PCNA-ATP\gammaS complex with DNA. However, the possibility remained that PCNA unloading requires ATP hydrolysis and thus is not supported by ATP\gammaS. The fact that ATP\gammaS induced the

**Figure 5.** DNA binds in the central chamber of RFC. A, the model of RFC-DNA shows only the Rfc2–4 subunits and the conserved positively charged residues located in the central cavity of RFC. The residues listed to the right are among the conserved residues and were each substituted with alanine. B, the RFC mutant (red squares) was analyzed for DNA binding using a rhodamine-labeled primer-template DNA as described under "Experimental Procedures." DNA binding by RFC$_{1,4N}$ is shown for comparison (blue circles).
RFC DNA-binding mutant to unload PCNA from DNA may be explained by its ability to open PCNA yet inability to bind DNA, thereby leading to dissociation of the RFC open PCNA-ATPγS complex from DNA. These observations indicate that the PCNA-opening step does not require ATP hydrolysis. Thus, it may be presumed that RFC uses ATPγS to open PCNA and to bind DNA, but that hydrolysis is needed to break the grip of RFC on DNA, allowing it to eject from the PCNA-DNA complex.

Rad24-RFC Is an Efficient PCNA Unloader—The alternative Rad-RFC DNA damage response clamp loader functions with the 911 clamp (37–39). Rad-RFC lacks Rfc1; it consists of the Rfc2–5 subunits in combination with Rad24 (Rad17 in humans), which replaces Rfc1 (see diagram in Fig. 7). Previous studies (37) have shown that Rad24-RFC does not load PCNA onto DNA, but that the Rad-RFC ATPase is stimulated by PCNA. Thus, the Rad-RFC complex must interact with PCNA even though it does not load PCNA onto DNA.

Does Rad-RFC open the clamp when it binds PCNA? To test this possibility, 32P-PCNA was loaded onto a singly nicked plasmid DNA and purified from free 32P-PCNA, RFC, and ATP by gel filtration. The 32P-PCNA-DNA complex was treated with Rad-RFC or the equivalent volume of buffer, and then the reaction was analyzed by gel filtration a second time to determine whether 32P-PCNA was unloaded from DNA. Rad-RFC removed most of the 32P-PCNA from DNA (Fig. 7B). To evaluate the efficiency of PCNA unloading by Rad-RFC, the experiment was repeated using different concentrations of Rad-RFC. The results show that Rad-RFC was quite efficient and was half-maximal at ~50 nM. The results with Rad-RFC are also consistent with the other experiments in this study demonstrating that Rfc1 is not required for clamp opening.

**FIGURE 6.** ATP binding is sufficient for clamp opening by RFC. A, clamp loading assays. The RFC DNA-binding mutant was tested for ability to load PCNA onto DNA (blue squares). Analysis of PCNA loading by RFCΔN is shown for comparison (red circles). The presence of the RFC DNA-binding mutant inhibited clamp loading by RFCΔN (green triangles). Reactions contained 100 nM RFCΔN and/or RFC DNA-binding mutant. B, clamp unloading assays. Right panel, the RFC DNA-binding mutant could unload PCNA in the presence of either ATP (blue squares) or ATPγS (red circles). A control reaction carried out in the absence of ATP is also shown (green triangles). Left panel, RFCΔN utilized ATP, but not ATPγS, for PCNA unloading. Unloading reactions contained 100 nM RFC.
PCNA clamp to bind all five RFC subunits (34). A recent electron microscopic reconstruction of archaeal RFC bound to PCNA provides experimental support for this conclusion (35). In addition, a recent fluorescence resonance energy transfer study of yeast RFC-PCNA interaction indicates that PCNA is held open by RFC in response to bound nucleotide (40). Surface plasmon resonance measurements in this study demonstrated that Rfc2 and Rfc5 both interact with PCNA. We presume that Rfc5 binds to the hydrophobic pocket between the PCNA domains of the third protomer and that, like Rfc4, Rfc2 binds to an interface between PCNA protomers.

**Eukaryotic Versus Prokaryotic Clamp Opening**—The Rfc1 subunit of the clamp loader is in the position analogous within the heteropentamer to the δ wrench of the *E. coli* γ complex. However, the *E. coli* δ wrench can open the *E. coli* β clamp and unload it from DNA by itself (20). δ plugs into a hydrophobic pocket of β and interacts with the interface, distorting it to a form that is incompatible with ring closure (21). In contrast, the studies described herein indicate that the spatially analogous Rfc1 subunit is not needed for clamp opening. The evidence is as follows. 1) An RFC mutant containing an Rfc1 subunit deleted in the PCNA-interacting AAA+ region retains function in PCNA clamp unloading. 2) An Rfc2:3:4:5 complex (no Rfc1) is capable of unloading PCNA from DNA. 3) The RFC:PCNA:ATP:γS structure shows extensive interaction of Rfc1 with PCNA, yet the PCNA ring remains shut. 4) Finally, the RadRFC DNA damage checkpoint response clamp loader, which lacks Rfc1, opens PCNA and unloads it from DNA nearly as efficiently as RFC1,2,3,4,5. The clamp-interacting helix of Rfc1, unlike that of δ, does not interact with or disrupt the PCNA interface as observed for the δ wrench.

This study demonstrates that the eukaryotic RFC clamp loader requires subunits other than Rfc1 for PCNA clamp opening. In particular, Rfc5 is required to open PCNA. Of the three hydrophobic pockets in PCNA, the only one that is not filled in the crystal structure is the pocket positioned below Rfc5. As the clamp is closed in the RFC:PCNA:ATP:δS structure, interaction of Rfc5 (and Rfc2) with PCNA may be needed to open PCNA. Furthermore, the interface of PCNA that opens for clamp loading onto DNA is presumed to be the interface positioned below the Rfc1 and Rfc5 subunits because DNA must pass between these two subunits to gain access to the central chamber of the clamp loader. Hence, if Rfc5 destabilizes the PCNA ring, it seems reasonable to suggest that the PCNA clamp is opened from the opposite side of the clamp interface that the *E. coli* δ wrench acts upon (Fig. 8A).

The δ wrench can open β and unload it from DNA, but cannot load β onto DNA. Clamp unloading is an important cellular function, as clamps are used in stoichiometric fashion on the lagging strand (6, 22). One β clamp is required for each Okazaki fragment, and as each Okazaki fragment is completed, the clamp is left on DNA and must be recycled. The cellular excess of δ relative to the other subunits and the efficiency of δ in unloading β from DNA have led to the suggestion that the excess δ recycles β clamps from DNA as they are left behind by the moving replisome (41).

This study demonstrates that the Rfc2:3:4:5 and Rfc2:5 subcomplexes are capable of unloading PCNA clamps from DNA *in vitro*. Could these same subcomplexes exist *in vivo* to aid in the recycling of PCNA during replication? Measurements of the intracellular levels of the RFC subunits have been made in a global analysis of protein expression in yeast by Ghaemmaghami et al. (42). The copy numbers of Rfc5 (5040) and Rfc2 (4610) are about twice as abundant as those of Rfc1 (2360), Rfc3 (3140), and Rfc4 (2160). These intracellular copy numbers predict an excess of Rfc2:5 complex in the cell, leaving ~2000 copies of free Rfc2:5

**DISCUSSION**

**All Five RFC Subunits Bind PCNA**—Proteins that bind sliding clamps often do so via hydrophobic interaction in a binding pocket positioned between two globular domains of the clamp. This was initially demonstrated for p21cip1/WAF1 cell cycle inhibitor binding to human PCNA via the conserved PCNA-interacting peptide sequence within p21cip1/WAF1 (3). PCNA has three of these pockets, one in each PCNA monomer. The RFC:PCNA:ATP:γS structure reveals that Rfc1 and Rfc3 contain residues that project into this pocket on adjacent PCNA protomers (8). Located between Rfc1 and Rfc3, Rfc4 is positioned over the interface between these PCNA protomers, and the interaction with PCNA is relatively minor compared with Rfc1 and Rfc3.

The RFC:PCNA structure shows that Rfc2 and Rfc5 do not contact PCNA (8). Molecular simulations of PCNA opening indicate that the clamp protomers open out of plane to form a right-handed helix, which complements the helical arrangement of RFC and may allow an open

**FIGURE 7.** RadRFC is a PCNA clamp unloader. A, RadRFC (100 nm) was tested for ability to unload PCNA from DNA (green triangles). The buffer control is shown for comparison (red circles). B, RadRFC was titrated in separate clamp unloading reactions. The amount of PCNA-DNA complex remaining is plotted versus concentration of RadRFC used in each reaction.
complex to perform activities such as PCNA unloading. Assuming a 1-μm diameter of the yeast cell nucleus, 2000 copies of Rfc2-5 complex correspond to a concentration of ~1.9 μM. This value is within the range of the concentration of Rfc2-5 required in this study to observe PCNA unloading activity.

DNA Binds inside RFC—The structure of RFC-PCNA:ATPγS and the helical architecture of the central chamber provide a compelling argument for placement of DNA inside the RFC pentamer (8). Experimental support for the placement of DNA inside RFC is provided in this study. As a start in exploring RFC-DNA interaction, we have mutated conserved basic residues within the Rfc2–4 subunits that are predicted to bind DNA. We found herein that this “RFC DNA-binding mutant” has dramatically decreased affinity for DNA, providing experimental support for the model of the RFC-DNA complex.

Interestingly, the RFC DNA-binding mutant can use either ATP or ATPγS to open the clamp and unload PCNA from DNA. This non-hydrolyzable ATP analog typically leads to an RFC-PCNA complex that remains tightly stuck to DNA (17, 18). In light of the ability of the RFC DNA-binding mutant to unload PCNA with ATPγS, we expect that the tight attachment of the wild-type RFC:PCNA:ATPγS complex to DNA is mediated mainly through RFC-DNA contacts rather than PCNA-DNA interaction. It is possible that the PCNA ring is held in the open state in the ATPγS-RFC:PCNA-DNA complex because the affinity for DNA lies in RFC and not necessarily a topologically closed PCNA ring as an anchor. Indeed, a previous study demonstrated that the E. coli β clamp is open while attached to the γ complex and DNA in the presence of ATPγS (16). In addition, a recent electron microscopic image reconstruction of the archaeal RFC:PCNA complex showed that the PCNA ring is held open in the presence of ATPγS (35). A recent fluorescence resonance energy transfer study of yeast RFC-PCNA complex also demonstrates that PCNA is held in an open lockwasher configuration (40).

Rad24-RFC Damage Checkpoint Clamp Loader Unloads PCNA—The 911 heterotrimeric clamp is assembled onto DNA by the Rad-RFC clamp loader, in which Rfc1 is replaced by Rad24 protein (Rad17 in humans) (12). Although Rad-RFC is not known to load PCNA onto DNA, it has an intrinsic DNA-dependent ATPase activity that is stimulated by PCNA (37, 43). Hence, Rad-RFC must bind the PCNA clamp, although its function with PCNA is not known. We have shown here that Rad-RFC unloads PCNA from DNA. A yeast proteomic study of the intracellular copy number of proteins places the Rad24 protein at a level of 752 molecules/cell (42). At this level and assuming full formation with the other RFC subunits, Rad-RFC would exist in the nucleus at a concentration of ~710 nM. This level is 7-fold above the concentration needed to saturate the clamp unloading assays in this work. Hence, there may be sufficient amounts of Rad-RFC in the cell to participate in the recycling of PCNA clamps from DNA.

Other alternative clamp loaders that contain Rfc2–5 could also play a role in PCNA unloading. Ctf18-RFC is an alternative clamp loader involved in chromosome cohesion, and it is capable of loading PCNA onto DNA (44). A recent study demonstrated that only 3–10 nM Ctf18-RFC is needed to unload PCNA (33). Bylund and Burgers (33) did not observe PCNA unloading by either Rad-RFC or RFC1AN: The different results observed herein can be explained by the different conditions used in this study relative to those used in the earlier study. For example, the earlier study used much lower concentrations of clamp loader complexes (~10 nM), which, compared with this study, are or below the threshold levels needed to observe PCNA unloading activity.

It seems possible that PCNA clamp unloading could be accomplished by any of several different complexes. Perhaps different clamp loaders assort to different locales in the cell. For example, it has been suggested that Ctf18-RFC may unload PCNA when replication forks meet the cohesion apparatus (33). The Rad-RFC clamp loader is responsible for loading 911 clamps onto DNA in the damage checkpoint response. Rad-RFC may also clear pre-existing PCNA clamps from DNA, perhaps to make room for 911 clamps. An important role of the DNA damage checkpoint is to halt ongoing DNA synthesis. Hence, the inability of Rad-RFC to load PCNA and the ability to unload PCNA may contribute to replication shutdown during DNA damage.
DNA: Replication, Repair, and Recombination:
Mechanism of Proliferating Cell Nuclear Antigen Clamp Opening by Replication Factor C

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