

## ATP Utilization by Yeast Replication Factor C

### III. THE ATP-BINDING DOMAINS OF Rfc2, Rfc3, AND Rfc4 ARE ESSENTIAL FOR DNA RECOGNITION AND CLAMP LOADING\*

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Sonja L. Gary Schmidt, Xavier V. Gomes, and Peter M. J. Burgers‡

From the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

**The conserved lysine in the Walker A motif of the ATP-binding domain encoded by the yeast *RFC1*, *RFC2*, *RFC3*, and *RFC4* genes was mutated to glutamic acid. Complexes of replication factor C with a N-terminal truncation ( $\Delta 2$ –273) of the Rfc1 subunit (RFC) containing a single mutant subunit were overproduced in *Escherichia coli* for biochemical analysis. All of the mutant RFC complexes were capable of interacting with PCNA. Complexes containing a *rfc1-K359E* mutation were similar to wild type in replication activity and ATPase activity; however, the mutant complex showed increased susceptibility to proteolysis. In contrast, complexes containing either a *rfc2-K71E* mutation or a *rfc3-K59E* mutation were severely impaired in ATPase and clamp loading activity. In addition to their defects in ATP hydrolysis, these complexes were defective for DNA binding. A mutant complex containing the *rfc4-K55E* mutation performed as well as a wild type complex in clamp loading, but only at very high ATP concentrations. Mutant RFC complexes containing *rfc2-K71R* or *rfc3-K59R*, carrying a conservative lysine  $\rightarrow$  arginine mutation, had much milder clamp loading defects that could be partially (*rfc2-K71R*) or completely (*rfc3-K59R*) suppressed at high ATP concentrations.**

Replication factor C (RFC)<sup>1,2</sup> uses the energy of ATP hydrolysis to load PCNA onto a primer-template junction. Sequence comparison studies indicate that each of the five Rfc subunits has an ATP-binding domain (reviewed in Ref. 1). The ATP-binding motif present in each of these five subunits represents a structural domain that may also function in ATP binding and/or hydrolysis. The prototypic structure for this domain is the *Escherichia coli*  $\delta$  subunit of the  $\gamma$ -complex (2, 3). The

structure of  $\delta$  is C-shaped with the Walker A and B motifs situated at the base near the hinge of the C. Although it has imperfect ATP-binding motifs and does not bind ATP, the  $\delta$  protein has high sequence similarity to the  $\gamma$  subunit, the active ATPase of the  $\gamma$ -complex, and therefore the structures of these two proteins are expected to be similar. Sequence comparisons between the five RFC subunits and  $\delta$  suggest that they may have a similar structure, at least in the base and hinge of the C. From this structure one can easily visualize how ATP binding could cause a conformational change in the entire protein. The top and base of the C clamp are only connected by a small hinge region, giving the overall structure some flexibility. ATP binding could result in an opening or closing of the C, which could be transmitted to the other subunits within the complex, resulting in an overall conformational change in RFC or the  $\gamma$ -complex (2).

The studies in the previous paper (4) indicate that RFC can bind up to four molecules of ATP depending on the presence of PCNA and primer-template DNA. In agreement with these experimental results are sequence comparison studies indicating that the Rfc1–Rfc4 subunits contain consensus ATP-binding motifs (see Fig. 1). The Walker A consensus sequence is GXXXXGKT, in which the lysine is invariant (5). This lysine coordinates oxygens on the  $\beta$  and  $\gamma$  phosphates of ATP (6, 7). The B motif ( $\Phi\Phi\Phi\Phi\Phi\Phi\Phi\Phi$ ), in which  $\Phi$  is M, L, I, or V, is essential for chelation of the divalent metal ion (7). The Rfc1–Rfc4 subunits contain both consensus motifs, whereas the Rfc5 subunit lacks critical residues in both the A and B motifs (see Fig. 1). As with the  $\delta'$  subunit of the *E. coli*  $\gamma$ -complex, the ATP-binding domain in Rfc5p likely is of structural importance rather than to bind ATP (2).

The observation that four molecules of ATP bind to a loading-competent complex of RFC with PCNA and DNA does not necessarily imply that binding and/or hydrolysis of all four ATPs is required for clamp loading. Because the four-subunit Rfc2–5 core complex can form alternative complexes, e.g. with Rad24p, it may well be the case that ATP binding to some subunit(s) is related to these alternative functions rather than loading of PCNA (8). A mutational analysis of the ATP-binding domains of these subunits may address this issue. Recently, mutational studies of the ATP-binding sites of the subunits of human RFC have been carried out. In one study, mutation to alanine of the conserved lysine in the Walker A motif in each of the human Rfc subunits, except for human Rfc5, greatly diminished or almost completely abolished clamp loading (9). In another study, the same lysine was mutagenized to glutamic acid in all subunits, again showing severe defects both in complex stability and activity in all mutants, except for that in Rfc5 (10).

In this paper we present mutational studies of yeast RFC, in

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. E-mail: burgers@biochem.wustl.edu.

<sup>1</sup> The abbreviations used are: RFC, replication factor C; Rfcx, x<sup>th</sup> subunit of RFC; RFC-xE, RFC complex with Lys  $\rightarrow$  Glu mutation in x<sup>th</sup> subunit; Rfc2–5, complex of Rfc2p, Rfc3p, Rfc4p, and Rfc5p; PCNA, proliferating cell nuclear antigen; ATP $\gamma$ S, adenosine (3-thiotriphosphate); SPR, surface plasmon resonance; DTT, dithiothreitol; Pol, polymerase.

<sup>2</sup> The truncation derivative of replication factor C containing Rfc1- $\Delta$ (aa3–272) has been used in this biochemical study. For ease of reading, this complex has been simply designated as RFC, and the Lys  $\rightarrow$  Glu mutant complexes have been designated as RFC-1E, RFC-2E, RFC-3E, and RFC-4E.

which we have mutated the conserved lysine in the Walker A motif of the *RFC1*, *RFC2*, *RFC3*, and *RFC4* genes to glutamic acid and also, for the *RFC2* and *RFC3* genes, to arginine. Whereas the Lys → Glu mutations in Rfc2p and Rfc3p have strong adverse effects on DNA binding, ATPase activity, and clamp loading activity defects in RFC containing a mutant (Lys → Glu) Rfc4 subunit were only apparent at a low ATP concentration. Surprisingly, no significant catalytic defect could be ascribed to the Rfc1 (Lys → Glu) mutation, but the stability of the mutant RFC complex was compromised.

#### EXPERIMENTAL PROCEDURES

**Strains and Plasmids**—For protein overproduction *E. coli* strains BL21(DE3)pLysS and BL21-CodonPlus(DE3)-RIL (Stratagene) were used. PY90-R (*MAT $\alpha$*  *rfc2 $\Delta$ ::kanMX6 ade2-1 his3-11, 15 ura3-1 trp1-1 leu2-3, 112 can 1-100 + pBL621 (rfc2-K71R)*) and PY92-R (*MAT $\alpha$*  *rfc3 $\Delta$ ::kanMX6 ade2-1 his3-11, 15 ura3-1 trp1-1 leu2-3, 112 can 1-100 + pBL623 (rfc3-K59R)*) were used for overexpression in yeast.

A series of plasmids was created for overproduction of Rfc subunits in *E. coli* expression vector pPY55. The genes were under the control of the T7 RNA polymerase promoter and the T7 phage gene 10 ribosome binding site and leader sequence. pBL482 (T7prom-*rfc1- $\Delta$*  (3–272) *pA-CYCori Kan<sup>R</sup>*) is an *E. coli* expression plasmid containing an N-terminal truncated version of *RFC1* (11). pBL482-E (T7prom-*rfc1-K359E,  $\Delta$*  (3–272)) was created by cloning a 524-base pair *NcoI/PvuII* fragment from pBL642-E (31) into the *NcoI/PvuII* sites of pBL482. pBL565 has been described (11). pBL565-E (T7prom-*rfc2-K71E Amp<sup>R</sup>*) was created by replacing the *PflMI/NheI* fragment of pBL565 (T7prom-*RFC2 Amp<sup>R</sup>*) with the same fragment from pBL622 (31). pBL456 (T7prom-*RFC3 Amp<sup>R</sup>*) has been described previously (12). pBL456-E (T7prom-*rfc3-K59E Amp<sup>R</sup>*) was created by replacing the 793-base pair *NcoI/EcoNI* fragment of pBL456 with the same fragment from pBL624 (31). pBL555 (T7prom-*RFC4 Amp<sup>R</sup>*) has been described previously (13). pBL555-E (T7prom-*rfc4-K55E Amp<sup>R</sup>*) was made by swapping in a 361-base pair *NcoI* fragment from pBL626 (31).

pBL481 is an *E. coli* expression plasmid that contains *rfc1- $\Delta$*  (3–272), *RFC2*, *RFC3*, *RFC4*, and *RFC5*, each under control of the T7 promoter and leader sequence (11). A series of mutant pBL481 plasmids, each containing a single mutant and four wild type *RFC* genes, was created by appropriate exchange of restriction fragments. These plasmids are named pBL481-1E (T7prom-*rfc1-K359E,  $\Delta$* (3–272) T7prom-*RFC2* T7prom-*RFC3* T7prom-*RFC4* T7prom-*RFC5 Amp<sup>R</sup>*), and pBL481-2E, pBL481-3E, and pBL481-4E, defined analogously.

pBL424 (*RFC1*, *RFC3*, *RFC4*, and *RFC5*), pBL425 (*RFC1*, *RFC2*, *RFC4*, and *RFC5*), pBL412 (*RFC2*), and pBL413 (*RFC3*) are multicopy *Saccharomyces cerevisiae* expression plasmids that contain the indicated *RFC* genes, each under control of the galactose-inducible *GAL1-10* promoter (14). pBL412-R and pBL413-R have the wild type genes swapped for *rfc2-K71R* and *rfc3K59R*, respectively.

**Purification of Individual RFC Subunits**—BL21(DE3)-pLysS cells containing pBL456, pBL456-E, pBL555, pBL555-E, pBL565, or pBL565-E were grown in 1 liter of Terrific Broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH<sub>2</sub>PO<sub>4</sub>, 72 mM K<sub>2</sub>HPO<sub>4</sub>) at 37 °C. At an A<sub>595</sub> of 0.7–1.0, the cells were induced for 3 h with 0.4 mM isopropyl- $\beta$ -thiogalactoside. The cells were harvested and resuspended in an equal volume of 50 mM Tris-HCl, pH 8.1, 10% sucrose and frozen. An equal volume of 2 $\times$  lysis buffer (1 $\times$  lysis buffer = 50 mM Tris-HCl, pH 8.1, 2 mM EDTA, 0.2 mM EGTA, 2  $\mu$ M leupeptin, 0.2  $\mu$ M pepstatin A, 5 mM NaHSO<sub>3</sub>, 3 mM DTT, 0.6 mg/ml lysozyme) was added upon thawing. Nonidet P-40 and phenylmethylsulfonyl fluoride were added to 0.05% and 1 mM final concentrations, respectively. After 10 min of incubation on ice, the lysate was briefly sonicated to reduce the viscosity. The lysate was spun for 20 min at 16,000 rpm in an SS-34 rotor. The insoluble pellet was washed twice with buffer B (buffer A, but with Tris-HCl, pH 7.5, and 2 M NaCl) and resuspended in buffer C (buffer B, but with 400 mM NaCl and 6 M urea). The samples were gently agitated at 4 °C for an hour and then spun at 16,000 rpm in an SS-34 rotor. The supernatant was retained and protein-renatured. The samples were diluted to 0.1–0.2 mg/ml in buffer D containing 5 M urea (buffer D contains 25 mM potassium phosphate, pH 7.2, 1 mM EDTA, 10% glycerol, 2  $\mu$ M pepstatin A, 2  $\mu$ M leupeptin, 5 mM NaHSO<sub>3</sub>, 3 mM DTT, 100 mM NaCl, 0.05% Brij 35) and then dialyzed twice for 3 h each time against 3 volumes of buffer D lacking urea. This usually resulted in 50–95% of the protein remaining soluble. After a clearing step in the centrifuge, the soluble Rfc subunits were concentrated by batch adsorption to Affi-Gel-Blue and step elution with 1 M NaCl in buffer D (lacking

urea). The samples were dialyzed against buffer D containing 100 mM NaCl and filtered to remove aggregates using Whatman Anotop 10 inorganic membrane filters with a 0.1- $\mu$ m pore size.

**Overproduction and Purification of Mutant RFC Complexes from *E. coli***—Three liters of cells were grown in Terrific Broth at 25–26 °C. The CodonPlus(DE3)-RIL cells contained either pBL481 or pBL481-1E, pBL481-2E, pBL481-3E, or pBL481-4E. When cells reached an A<sub>595</sub> of 1.8–2.5, they were induced with 0.4 mM isopropyl- $\beta$ -thiogalactoside and grown with shaking for another 6–8 h at 25–26 °C. The cells were harvested, resuspended in about 40 ml of 50 mM Tris-HCl, pH 8.1, 10% sucrose and frozen in liquid nitrogen. Lysis and sonication were as described above. After centrifugation at 16,000 rpm for 30 min, 5 M NaCl and 10% Polymin P were added to the supernatant to 250 mM and 0.04%, respectively, to precipitate the nucleic acids. After 15 min on ice, with periodic agitation, the mixture was spun for 20 min at 16,000 rpm. Ammonium sulfate was added to the supernatant to 0.28 gm/ml, which was stirred at 4 °C for 1 h followed by a spin at 16,000 rpm for 30 min. The pellet was resuspended in buffer A (25 mM KPO<sub>4</sub>, pH 7.2, 10% glycerol, 1 mM EDTA, 2  $\mu$ M pepstatin A, 2  $\mu$ M leupeptin, 3 mM DTT, 5 mM NaHSO<sub>3</sub>, 0.01% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) until the conductivity was equal to that of buffer A<sub>150</sub> (buffer A with 150 mM NaCl; Salt concentrations of the buffers (in mM NaCl) are indicated with a subscript). This is fraction I (usually about 90 ml). Fraction I was then loaded onto a 25-ml S-Sepharose column. After a 50-ml wash with buffer A<sub>150</sub>, the RFC was eluted with buffer A<sub>850</sub>. The RFC-containing fractions were combined (fraction II, about 30 ml) and dialyzed against buffer MA (50 mM Tris-HCl, pH 7.7, 0.5 mM EDTA, 10% glycerol, 8 mM magnesium acetate, 0.5 mM ATP, 5  $\mu$ M pepstatin A, 5  $\mu$ M leupeptin, 3 mM DTT, and 5 mM NaHSO<sub>3</sub>) until the conductivity had reached that of buffer B<sub>200</sub>. ATP was added to the dialyzed fraction to a final concentration of 1 mM. The sample was gently agitated for 1 h with 3–4 ml of PCNA-agarose beads equilibrated in B-200. The beads were packed into a column and washed with 6 ml of buffer MA<sub>200</sub> followed by 5 ml of buffer MA<sub>300</sub>. The protein was eluted with buffer E (30 mM Tris-HCl, pH 7.7, 2 mM EDTA, 10% glycerol, 5  $\mu$ M pepstatin A, 5  $\mu$ M leupeptin, 3 mM DTT, 5 mM NaHSO<sub>3</sub>, 0.05% ampholytes 3.5–9) plus 300 mM NaCl. The RFC containing fractions were pooled (fraction III, 10–12 ml) and dialyzed against buffer H (30 mM Hepes, 10% glycerol, 1 mM EDTA, 2  $\mu$ M pepstatin A, 2  $\mu$ M leupeptin, 3 mM DTT, 5 mM NaHSO<sub>3</sub>, 0.01% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) plus 150 mM NaCl. The samples were then run on a 1-ml MonoS column with a linear gradient of 100–600 mM NaCl in buffer H.

**Overproduction and Purification of Mutant RFC Complexes from Yeast**—To obtain mutant complexes from yeast, strain PY90-R (containing chromosomal *rfc2-K71R*) was transformed with plasmids pBL424 and pBL412-R, and strain PY92-R (containing chromosomal *rfc3-K59R*) was transformed with plasmids pBL425 and pBL413-R. The strains will be described elsewhere (31). Yeast growth, induction by galactose, and purification of mutant RFC-2R and RFC-3R complexes, respectively, were exactly as described for wild type (14).

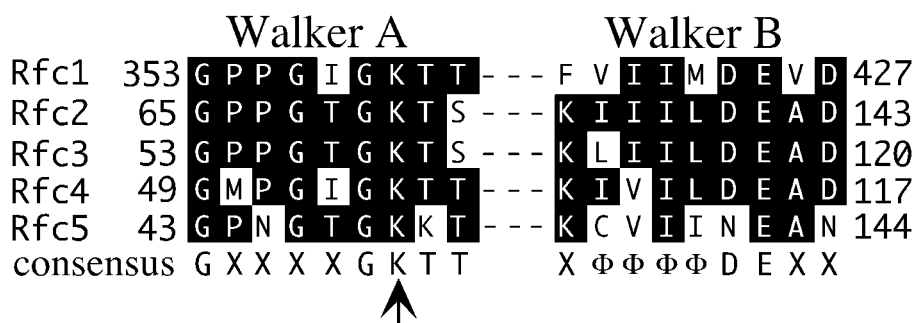
**ATPase Activity Assays**—ATPase activity assays were carried out as described (4). Initial rates of hydrolysis of [ $\alpha$ -<sup>32</sup>P]ATP were determined from time courses at increasing ATP concentrations. *K<sub>m</sub>* and *V<sub>max</sub>* values were obtained by fitting the data to a one-site Michaelis-Menten model.

**Pol  $\delta$  Holoenzyme DNA Replication Assays**—The standard 30- $\mu$ l mp18 DNA replication assay contained 40 mM Tris-HCl, pH 7.8, 8 mM MgAc<sub>2</sub>, 0.2 mg/ml bovine serum albumin, 75 mM NaCl, 1 mM DTT, 0.1% ampholytes 3.5–9.5, 100  $\mu$ M each dCTP, dGTP, and dTTP, 12.5  $\mu$ M [ $\alpha$ -<sup>32</sup>P]dATP, 1 mM ATP, 40 fmol (100 ng) of singly primed single-stranded mp18 DNA, 850 ng of *E. coli* single-stranded binding protein, 50 fmol (25 ng) of Pol  $\delta$ , 1 pmol (90 ng) of PCNA as trimers, and the indicated amounts of RFC. The aliquots were quenched in half the volume of 50 mM EDTA, 1% SDS, 50% glycerol, 0.05% bromphenol blue, and the products were separated on 0.8% alkaline agarose gels and subjected to autoradiography.

The standard 50- $\mu$ l poly(dA)-oligo(dT) assay contained 40 mM Tris-HCl, pH 7.8, 8 mM MgAc<sub>2</sub>, 0.2 mg/ml bovine serum albumin, 75 mM NaCl, 1 mM DTT, 0.1% ampholytes 3.5–9.5, 30  $\mu$ M [<sup>3</sup>H]dTTP, 250 ng of poly(dA)-(dT)<sub>22</sub> (40:1 nucleotide ratio), 1.6  $\mu$ g of *E. coli* single-stranded binding protein, 2 pmol of PCNA, 100 fmol Pol  $\delta$ , and ATP and RFC as indicated. After incubation at 30 °C for 8 min, acid-precipitable radioactivity was determined. The chain length of poly(dA) was ~300.

**Bi-molecular Interaction Analysis**—Surface plasmon resonance (SPR) was performed on a BIAcore X apparatus. The running buffer used in the analysis was 30 mM Hepes-NaOH, pH 7.5, 0.5 mM EDTA, 10% glycerol, 10 mM magnesium acetate, 125 mM sodium chloride, 0.1% ampholytes 3.5–9.8, and 0.01% Nonidet P-40, 0.2 mg/ml bovine serum

FIG. 1. The ATP-binding domains of the five RFC subunits. Consensus Walker A and Walker B domains are shown.  $\Phi$  indicates I, L, V, or M. The arrow indicates the conserved lysine mutated in this study to arginine or glutamic acid.



albumin. The DNA chips and PCNA chips were as described (15). The interaction of (mutant) RFC with PCNA and with DNA was monitored by injecting 90  $\mu$ l of the indicated concentrations of (mutant) RFC over a PCNA chip or a DNA chip at a flow rate of 30  $\mu$ l/min. Linear initial rates of binding were determined from the response curves between 4 and 15 s to avoid variations because of injection spikes at  $t = 0$  s.

## RESULTS

**Overproduction of Mutant RFC Complexes**—To determine which subunits of RFC were required for the ATPase and clamp loading activities of this complex, we mutated the conserved lysine in the Walker A motif of the *RFC1*, *RFC2*, *RFC3*, and *RFC4* genes to glutamic acid (Fig. 1). To examine the biochemical properties of RFC containing these mutations, five-subunit complexes were overproduced in *E. coli* using a plasmid system described previously (11). In this overproduction system we also used a truncation derivative of the Rfc1 subunit that deletes the ligase homology domain (Rfc1- $\Delta$  (3–272)). The truncation-containing complex eliminates ATP-independent DNA binding attributed to the ligase homology domain. The truncation derivative has the same clamp loading activity as the full-length complex and, because of the elimination of a competing DNA binding domain, may show increased activity in some assay systems (11, 16, 17). Each mutant five-subunit complex contained four wild type subunits and one mutant subunit. The mutant RFCs were designated RFC-1E (*i.e.* RFC containing Rfc1- $\Delta$  (3–272), K359E, Rfc2, Rfc3, Rfc4, and Rfc5), RFC-2E (RFC with Rfc2-K71E), RFC-3E (RFC with Rfc3-K59E), and RFC-4E (RFC with Rfc4-K55E). Limited biochemical studies were also carried out with mutant complexes in which conservative lysine  $\rightarrow$  arginine mutations were made in the *RFC2* and *RFC3* genes to yield RFC-2R (RFC with Rfc2-K71R) and RFC-3R (RFC with Rfc3-K59R), respectively, and which were overproduced and purified from yeast (14).

**Purification of Mutant RFC Complexes by PCNA-agarose**—Purification involved the use of a PCNA affinity column. Because salt-stable ATP-dependent binding to PCNA-beads requires the five-subunit enzyme (14, 15), binding of a mutant RFC in the presence of  $Mg^{2+}$ -ATP and subsequent elution by EDTA indicates that the mutant RFC assembled properly into a complex without major structural problems. Of course this rationale would not apply to a mutant complex that failed to bind PCNA because of failure to bind ATP to a subunit critical for this interaction. After an initial S-Sepharose batch purification step of the *E. coli* lysate, the mutant complexes were subjected to PCNA-agarose chromatography (see “Experimental Procedures”). The RFC-2E and RFC-3E mutant complexes bound as efficiently as wild type RFC to the PCNA-agarose beads in the presence of 1 mM ATP, 8 mM  $MgAc_2$ , and 300 mM NaCl. A considerable fraction (30–70% in several preparations) of the RFC-4E complex and an even larger fraction (50–80% in two preparations; see below) of the RFC-1E complex failed to bind to the affinity matrix, indicative that not all five-subunit complexes are fully active. However, when the RFC-1E complex that had bound to PCNA-agarose was subjected to a sec-

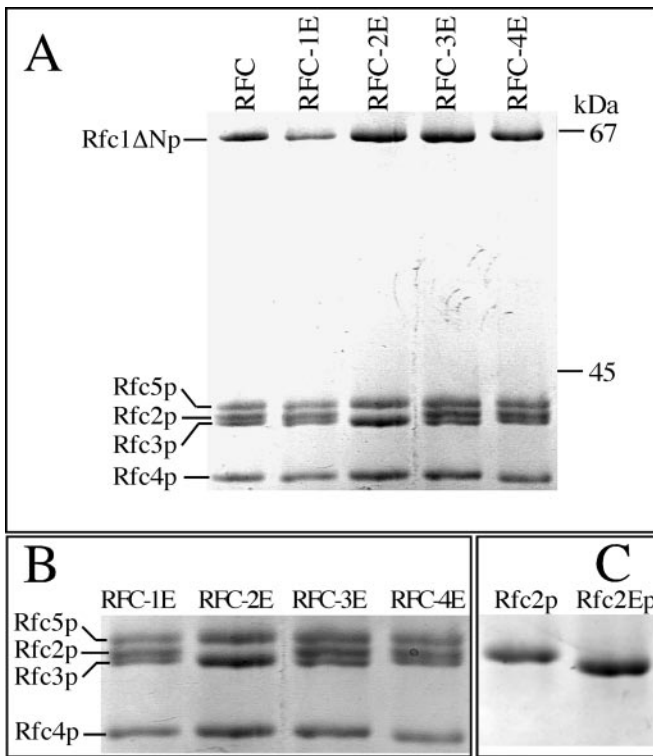
ond PCNA-agarose step, all RFC-1E complex bound. These data indicate that some fraction of the five-subunit complexes obtained from *E. coli* was inactive. Therefore, the PCNA-agarose step is a crucial purification step in our methodology, because it not only removes the excess of Rfc2–5 complex present in the lysates but also (mutant) five-subunit RFC complexes that were inactive because of misfolding or misassociation.

Purification of the RFC-1E complex posed a major difficulty because of rampant proteolysis of the Rfc1-E subunit. Four of the six independent RFC-1E overproduction and purification preparations we carried out resulted in complete proteolysis of the Rfc1-E subunit after the first column step. A 62-kDa species was a major intermediate of proteolytic breakdown, suggesting that the Lys  $\rightarrow$  Glu mutation at amino acid 93 of Rfc1 (the truncated version lacking the ligase homology domain)<sup>2</sup> resulted in a destabilization of the N-terminal domain of this subunit, which led to an initial proteolytic removal of  $\sim$ 40 amino acids. Such an N-terminal proteolytic truncation would reach into motif II of Rfc1, which is essential for RFC activity (16).<sup>3</sup> Of the two remaining preparations of RFC-1E, more than 90% of the Rfc1-E subunit was proteolyzed. However, a small fraction of Rfc1-E remained intact. When these preparations were subjected to PCNA-agarose, only a minor fraction (20–50%) bound and was eluted with EDTA. However, a large excess of Rfc2–5 still remained in the preparations, and the bound fraction was passed again over a PCNA-agarose column. In this second column all Rfc1-E containing material bound in the presence of  $Mg^{2+}$ -ATP and was eluted with EDTA. Even then, the subunit composition of this complex was not stoichiometric with a  $\sim$ 50% excess of small subunits remaining in the final preparation after MonoS column chromatography. Because of these problems with obtaining sufficient quantities of pure RFC-1E with proper subunit stoichiometry, studies with this complex were limited in scope.

Following purification, the complexes were examined by SDS-polyacrylamide gel electrophoresis. Fig. 2A shows the purified mutant RFCs, whereas a larger view of the small subunit region of the gel is shown in Fig. 2B. Except for RFC-1E (see above), all five subunits in the purified complexes were present in approximately equimolar amounts. The glutamic acid-containing subunits migrated slightly faster through the gel than the corresponding wild type subunits. This is seen most dramatically in the case of the Rfc2-E subunit, which co-migrated with Rfc3p, giving the appearance of the presence of only four subunits in the RFC-2E complex. The anomalous migration of Rfc2-Ep was confirmed by separate purification of the Rfc2 and Rfc2-E subunits and analysis by SDS-polyacrylamide gel electrophoresis (Fig. 2C).

**RFC Mutants Are Proficient for Binding PCNA**—The observation that all mutant RFCs could be purified over a PCNA-agarose column in the presence of  $Mg$ -ATP indicates that the

<sup>3</sup> X. V. Gomes and P. M. J. Burgers, unpublished results.

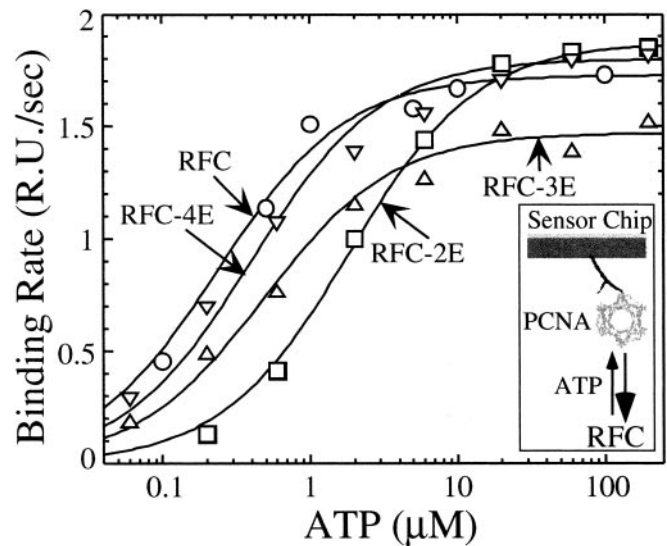


**FIG. 2. SDS-polyacrylamide gel electrophoresis of mutant RFC complexes.** *A*, wild type and mutant RFC complexes following purification. The gel was stained with colloidal Coomassie. *B*, enlarged view of the small subunit region. *C*, migration of the individually purified Rfc2p subunit and the mutant Rfc2Ep subunit.

ability to form a stable RFC-PCNA complex remains intact in the mutants (see above). To assess the quantitative requirement for ATP in the formation of a stable RFC-PCNA complex, we used SPR. Binding of RFC to a PCNA chip is greatly enhanced in the presence of ATP (4). The linear initial rate of binding to the chip is dependent on the concentration of active complexes, *i.e.* those RFC molecules that have the required number of ATP molecules bound to form a high affinity complex with PCNA on the chip. Therefore, by varying the ATP concentration at a constant concentration of RFC, the  $K_D$  for binding of ATP can be determined from the initial binding rates (4). The half-maximal response for wild type RFC was obtained at 0.5  $\mu\text{M}$  ATP. Surprisingly, RFC-2E, RFC-3E, and RFC-4E all yielded very similar  $K_D$  values of 0.5–2.2  $\mu\text{M}$  ATP (Fig. 3). The lower rate of binding for RFC-3E at saturating ATP probably reflects the presence of inactive complex in the preparation because the extent of RFC-3E binding at equilibrium was also 20–30% lower than that of wild type (data not shown).

**RFC-2E and RFC-3E Are Defective for DNA Binding**—SPR was also used to assess binding of the RFC mutants to DNA. As in all of these mutant RFCs the ligase homology domain of Rfc1 is deleted by an N-terminal truncation, binding of RFC to DNA is absolutely dependent on the presence of a primer-template junction and ATP $\gamma$ S (15). Injection of either wild type RFC or RFC-4E across a DNA chip in the presence of 1 mM ATP $\gamma$ S yielded a very similar sensorgram, indicating that DNA binding by RFC-4E was not impaired under these conditions. However, binding of RFC-3E and particularly RFC-2E was severely impaired (Fig. 4A).

Injection of RFC together with PCNA and ATP $\gamma$ S over the DNA chip allows the detection of complexes in which PCNA has been loaded by RFC, but the termination step, release of RFC, is blocked because hydrolysis of bound ATP $\gamma$ S does not occur (15). A robust signal was observed when either wild type RFC



**FIG. 3. Mutant RFCs bind PCNA.** SPR was used to determine linear initial rates of binding of 30 nM (mutant) RFC to a PCNA chip as a function of the ATP concentration. The data were fitted to a one-site saturation binding model. The inset shows a diagram of the experiment.

or RFC-4E together with PCNA and 1 mM ATP $\gamma$ S were flowed across the chip (Fig. 4B). The signal obtained with RFC-3E was barely above background (the no nucleotide control). It was also much weaker than that obtained in the absence of PCNA (Fig. 4A). Even more striking was the result with RFC-2E, which had shown very weak binding in the absence of PCNA but showed absolutely no binding to DNA in the presence of PCNA.

The signals obtained with RFC-2E and RFC-3E were too low to permit more detailed studies. For the RFC-4E mutant we determined the  $K_D$  value for ATP $\gamma$ S required to establish a stable complex with DNA either in the absence or presence of PCNA. These values were 10–100-fold higher than for wild type RFC, suggesting that ATP binding to Rfc4p is required for stable DNA binding (Fig. 4C).

**ATPase Activity of the Rfc3 Subunit**—Rfc3 is the only subunit of RFC that has been shown to possess an intrinsic ATPase activity (12). This ATPase is stimulated about 10-fold by DNA, but primer-template junctions are not required for this stimulation. With a turnover number of  $\sim 0.02 \text{ s}^{-1}$ , this activity is much lower than that of the RFC complex (Table I). Mutant Rfc3 with either a K59R or K59E mutation were over-expressed and purified from *E. coli*. The DNA-dependent ATPase activity was determined at 50  $\mu\text{M}$  ATP. The turnover number of Rfc3 was  $0.019 \text{ s}^{-1}$ , that of Rfc3-K59R was  $0.005 \text{ s}^{-1}$ , and Rfc3-K59E was completely inactive ( $< 0.001 \text{ s}^{-1}$ ) (data not shown). The ATPase activity of Rfc3-K59R in the absence of DNA was close to background levels ( $\sim 0.001 \text{ s}^{-1}$ ).

**ATPase Activity of RFC Mutants**—Comparative ATPase studies were performed at 50  $\mu\text{M}$  ATP, a concentration close to saturation for wild type RFC (Fig. 5A) (4). As described before, the basal ATPase activity of wild type RFC is stimulated 2–3-fold by PCNA. Mutant RFC-2E and RFC-3E showed a very low basal ATPase, which was not stimulated by PCNA, even though both mutants bound PCNA efficiently (Fig. 3). In contrast, in the absence of DNA, the ATPase activity of RFC-4E with or without PCNA was indistinguishable from that of wild type (Table I).

Upon addition of primer-template DNA, the ATPase of RFC was stimulated 7–8-fold, and addition of PCNA gave a further 3-fold stimulation. A 2–3-fold stimulation by DNA of the RFC-2E ATPase was observed, but further addition of PCNA showed an inhibition rather than a stimulation of the ATPase, reflecting the DNA binding properties of this mutant complex

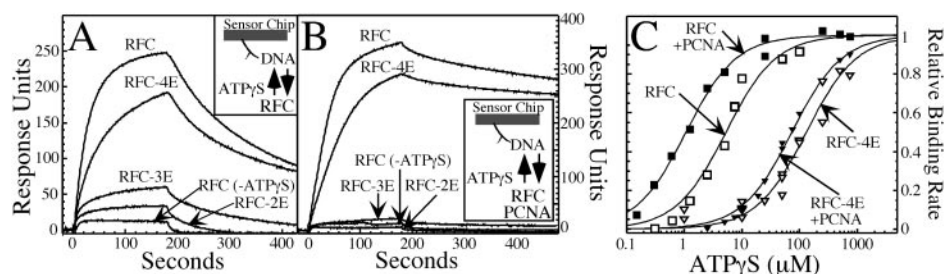


FIG. 4. **RFC-2E and RFC-3E are defective in DNA binding and PCNA loading.** A, SPR sensorgrams of binding of (mutant) RFC (30 nM) to a primed DNA chip (see “Experimental Procedures”) in the presence of 1 mM ATP $\gamma$ S. B, PCNA (120 nM) and (mutant) RFC (30 nM) were injected onto the DNA chip in the presence of 1 mM ATP $\gamma$ S. C, 30 nM RFC or RFC-4E with or without 120 nM PCNA were injected onto a DNA chip at increasing concentrations of ATP $\gamma$ S. Relative linear initial rates of binding ( $k_{on}/k_{on,max}$ ) were plotted against the ATP $\gamma$ S concentration, and the data were fitted to a one-site saturation binding model. The ATP $\gamma$ S  $K_D$  values were  $6 \pm 2 \mu\text{M}$  for RFC,  $1.1 \pm 0.5 \mu\text{M}$  for RFC+PCNA,  $140 \pm 40 \mu\text{M}$  for RFC-4E, and  $74 \pm 20 \mu\text{M}$  for RFC-4E with PCNA.

TABLE I  
ATPase activity of RFC and RFC-4E

The reactions were conducted in the presence or the absence of PCNA and/or multiply-primed DNA. When in the reactions, PCNA and/or DNA primer termini were present at a 2-fold molar excess compared with RFC. See “Experimental Procedures” for details.

Cofactor	RFC		RFC-4E	
	$K_m$ $\mu\text{M}$	$k_{cat}$ $\text{s}^{-1}$	$K_m$ $\mu\text{M}$	$k_{cat}$ $\text{s}^{-1}$
None	$3.1 \pm 0.8$	$0.07 \pm 0.02$	$2.6 \pm 0.6$	$0.08 \pm 0.02$
PCNA	$1.5 \pm 0.5$	$0.16 \pm 0.02$	$1.5 \pm 0.5$	$0.19 \pm 0.02$
DNA	$9.7 \pm 3.0$	$0.51 \pm 0.06$	$3.7 \pm 1.8$	$0.11 \pm 0.02$
PCNA/DNA	$8.4 \pm 1.4$	$1.56 \pm 0.1$	$40 \pm 10$	$0.57 \pm 0.06$

(Fig. 4, A and B). The ATPase activity of the RFC-3E complex was marginally stimulated by DNA. However, unlike observed with RFC-2E, the addition of PCNA resulted in a weak but significant stimulation of the ATPase activity, suggesting that RFC-3E may possess a weak clamp loading activity (Fig. 5A).

Considering the observed ATP defect in DNA binding for RFC-4E (Fig. 4C), we carried out a full kinetic analysis of this mutant complex (Fig. 5, B and C). The  $k_{cat}$  value of the DNA-stimulated RFC-4E ATPase was one third of that of wild type. However, the  $K_m$  value of the DNA-stimulated ATPase was only  $4 \mu\text{M}$ , which is much lower than the  $K_D$  value of  $140 \mu\text{M}$  for ATP $\gamma$ S necessary to form a stable complex with the DNA (compare Fig. 4C with Fig. 5B). However, because both the  $K_m$  and  $k_{cat}$  values of the RFC-4E ATPase are the same with or without DNA present (Table I), the data obtained in the presence of primer-template DNA likely reflect the activity of the complex without DNA. One might expect to see a further increase in ATPase activity at high ATP levels at which DNA binding by RFC-4E occurs, but no such increase was observed, even at  $300 \mu\text{M}$  ATP, the practical upper limit for carrying out these assays (Fig. 5B). On the other hand, the observed  $K_m$  for the DNA-stimulated ATPase of RFC-4E in the presence of PCNA is more in agreement with the binding data (compare Fig. 4C with Fig. 5C; Table I).

**ATP Defects in Clamp Loading**—Two series of replication assays were carried out to indirectly determine the efficiency of clamp loading by the mutant RFCs. Replication of poly(dA)-oligo(dT) by Pol  $\delta$  is stimulated by PCNA because PCNA can load by diffusion onto the ends of the linear DNA molecule (18, 19). However, when the NaCl concentration in the assay is raised to  $75 \text{ mM}$ , PCNA can no longer load by diffusion onto the DNA ends, and processive DNA replication by Pol  $\delta$  and PCNA is absolutely dependent on the presence of RFC and ATP. Therefore, this replication assay indirectly measures the efficiency of PCNA loading. However, because PCNA when loaded by RFC onto short polynucleotides such as poly(dA)-oligo(dT) can easily dissociate from the linear DNA by sliding off the end, efficient replication of these DNA templates by Pol  $\delta$  requires

multiple loading events of PCNA by RFC (11, 20, 21). On the other hand, one single event stably loads PCNA on primed single-stranded circular phage DNA.

At 1 mM ATP, RFC-1E and RFC-4E efficiently loaded PCNA onto poly(dA)-oligo(dT), resulting in efficient replication by Pol  $\delta$ , whereas inefficient but measurable DNA synthesis was observed at high RFC-3E levels. RFC-2E was completely inactive in this assay (Fig. 6A). For each RFC that showed activity, the quantitative requirement for ATP was measured at those levels of RFC at which the response was linear with RFC concentration (Fig. 6B). Consistent with all previous observations of wild type RFC, half-maximal activation was obtained at  $5 \mu\text{M}$  ATP. The apparent  $K_m$  value of  $85 \mu\text{M}$  for RFC-4E is also consistent with the other studies of this mutant (Figs. 4C and 5C). Surprisingly, RFC-1E was like wild type with an apparent  $K_m$  value of  $6 \mu\text{M}$  for PCNA loading. Finally, the low but measurable clamp loading activity of RFC-3E did allow us to determine an apparent  $K_m$  of  $320 \mu\text{M}$  ATP.

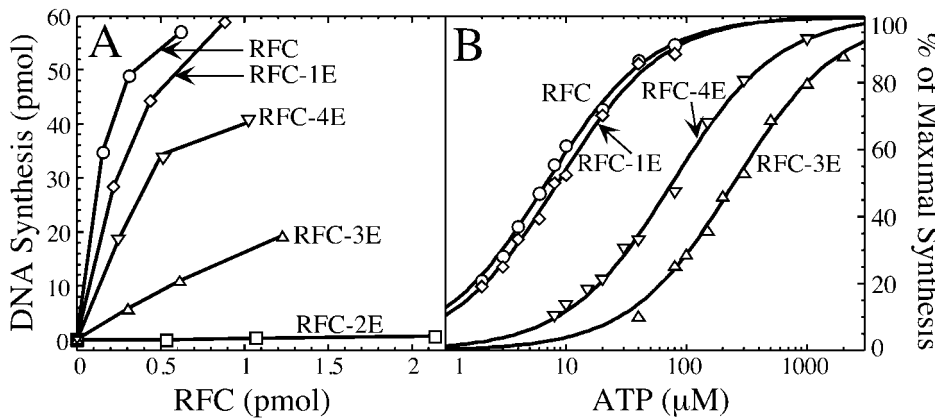
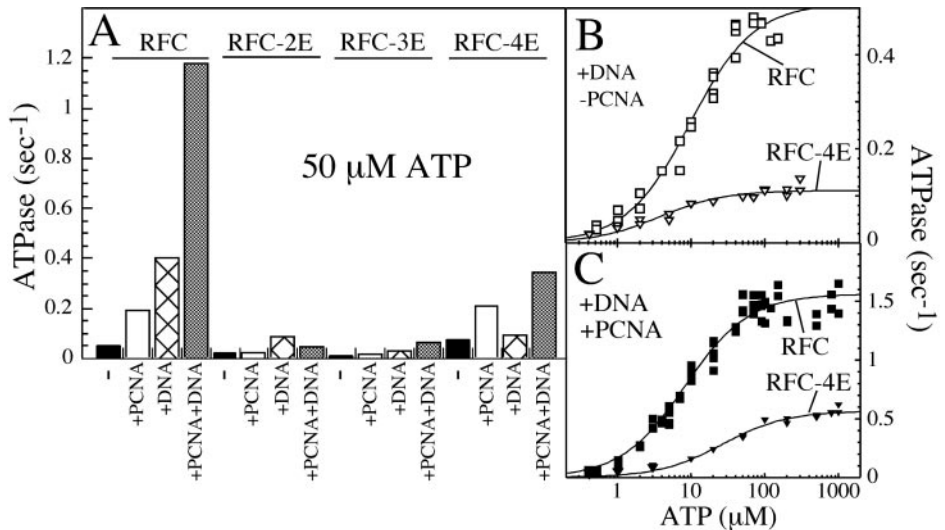
Replication of primed circular viral DNA templates, e.g. M13-mp18 DNA, follows a single loading event by RFC. To assess ATP concentration-dependent defects, the assay was carried out at either  $10 \mu\text{M}$  or 1 mM ATP. Full activity was observed for RFC-1E at both ATP concentrations, whereas RFC-4E showed reduced activity at  $10 \mu\text{M}$  ATP (Fig. 7A) but was fully active at 1 mM ATP (Fig. 7B). Again, the activity of RFC-3E was negligible at low ATP, but inefficient clamp loading could be detected at the high ATP concentration. Finally, RFC-2E was completely defective.

Finally, we have coupled PCNA loading to another read-out system. Activity of the FEN1 FLAP endonuclease at  $100 \text{ mM}$  NaCl is strictly dependent on PCNA encircling the DNA and therefore also on loading of the clamp by RFC (22). In this assay the results with the mutant RFCs were completely analogous to those obtained with the replication-coupled loading assays (data not shown).

**Clamp Loading Defects with Lysine  $\rightarrow$  Arginine Mutations in Rfc2p and Rfc3p**—Mutant complexes were also made with the conservative Lys  $\rightarrow$  Arg mutations in the Rfc2 and Rfc3 subunits. These mutant complexes were overproduced in yeast and contained the full-length Rfc1 subunit (see “Experimental Procedures”). As before, the mutant RFCs were tested for their ability to load PCNA indirectly using the primed M13-mp18 DNA replication assay (Fig. 8). These assays showed that RFC-3R<sup>4</sup> was fully functional at 1 mM ATP but showed defects at low ATP concentrations, much like the RFC-4E mutant discussed above. On the other hand, RFC-2R<sup>4</sup> was completely defective at low ATP and showed severe defects in loading even at 1 mM ATP. Considering our observation that substantial DNA-de-

<sup>4</sup> The Lys  $\rightarrow$  Arg mutant complexes contained the full-length Rfc1 subunit and are designated RFC-2R and RFC-3R.

**FIG. 5. ATPase activities of mutant RFC.** A, 25 ng of wild type RFC and up to 500 ng of mutant RFC was used in each assay which was performed at a fixed ATP concentration of 50  $\mu$ M. The data are expressed as mol of ATP hydrolyzed per mol of RFC/s. Standard errors of this analysis are 10% within a given set, *i.e.* for a particular mutant. B, DNA-stimulated ATPase activity of RFC and RFC-4E as a function of the ATP concentration. C, DNA- and PCNA-stimulated ATPase activity of RFC and RFC-4E as a function of the ATP concentration. PCNA and DNA when added were each in 2-fold molar excess. The ATP  $K_m$  and  $k_{cat}$  values are given in Table I. See "Experimental Procedures" for details.



**FIG. 6. ATP dependence of clamp loading.** DNA replication on poly(dA)-oligo(dT) was carried out as described under "Experimental Procedures." A, increasing levels of (mutant) RFC were used in a replication assay containing 2 pmol of PCNA and 0.1 pmol of Pol  $\delta$  for 8 min at 30 °C. Quantitation of RFC-1E was based on that of the large subunit. B, replication assays for 8 min at 30 °C as under A contained 0.1 pmol of RFC, 0.1 pmol of RFC-1E, 1.2 pmol of RFC-3E, or 0.2 pmol of RFC-4E, and increasing ATP. For each series of ATP titration curves, the  $V_{max}$  was calculated using Michaelis-Menten kinetics and set to 100% for illustrative purposes. ATP  $K_m$  values were  $5 \pm 2 \mu$ M for RFC,  $6 \pm 2 \mu$ M for RFC-1E,  $320 \pm 100 \mu$ M for RFC-3E, and  $85 \pm 20 \mu$ M for RFC-4E.

pendent ATPase activity remained in the isolated Rfc3-K59R subunit in comparison with wild type Rfc3, the observation of efficient clamp loading activity by the RFC-3R complex, especially at high ATP concentrations, was not that surprising.

DISCUSSION

*The ATP-binding Domains of Rfc2, Rfc3, and Rfc4 Are Required for PCNA Loading*—Our previous analysis of RFC showed that four molecules of ATP can bind to RFC under clamp loading conditions, *i.e.* with PCNA and primer-template DNA present. However, it did not address whether ATP binding to all four sites and their subsequent hydrolysis is actually required for loading of PCNA (4). The results of this study suggest that the ATP-binding sites of the Rfc2, Rfc3, and Rfc4 subunits, but not of the Rfc1 subunit, are required for clamp loading. However, these results need to be reconciled with very different results from mutational studies with human RFC (Table II).

The conserved lysine residue in the Walker A motif of ATP-binding proteins has been a favorite target for mutational studies. Mutation of this lysine to other residues, including glutamic acid, results in only slight alterations in the P loop structure as determined by x-ray crystallography and other methods (23–25). Therefore, defects associated with mutations in this residue have in general been ascribed to defects in ATP binding and/or hydrolysis rather than defects in protein folding or stability. The Rfc proteins are members of the AAA+ superfamily of ATP-binding proteins, which include many DNA-dependent ATPases such as the *E. coli* DnaA and RuvB proteins, the  $\gamma$  subunit of DNA polymerase III holoenzyme, and the eukaryotic Cdc6 and Mcm proteins (3). Mutation of the lysine

residue to isoleucine (dnaA), arginine (ruvB), alanine ( $\gamma$ -subunit), and glutamic acid (Cdc6) in all cases yielded a protein with  $\leq 1\%$  activity and, where measured, a more than 100-fold reduction in ATP binding affinity (26–29). Only in the human Mcm4,6,7 helicase, in which all three subunits have ATP-binding domains, was a partially active helicase recovered upon a Lys  $\rightarrow$  Ala mutation in Mcm6 (30).

Against this experimental background, the inactivity of the RFC-2E and RFC-3E complexes is easily understood; their ATP-binding domains are essential for one or more steps in clamp loading. Although the phenotype of RFC-4E is more unusual, the observation that full activity of this mutant complex could only be obtained at very high ATP concentrations still indicates that ATP binding to the Rfc4 subunit is essential for clamp loading.

*Mutations in Yeast and Human Rfc1 Have Opposite Phenotypes*—In contrast, our results with the mutant RFC-1E complex were very surprising because they appear to directly contradict analogous studies with human RFC (Table II) (9, 10). The only defect we detected in the mutant RFC complex containing Rfc1-K359E was its extreme sensitivity to proteolysis during isolation from *E. coli* extracts. However, we were able to obtain sufficient quantities to ascertain that the mutation did not affect clamp loading in coupled assays measuring DNA replication by Pol  $\delta$  (Figs. 6 and 7). Within the margin of error the apparent  $K_m$  value for ATP in PCNA loading by RFC-1E is identical to that of wild type RFC, and the  $V_{max}$  value is only slightly lower than that of wild type (Fig. 6).

With any mutational analysis there is the real possibility that loss of activity is obtained not because ATP binding to the

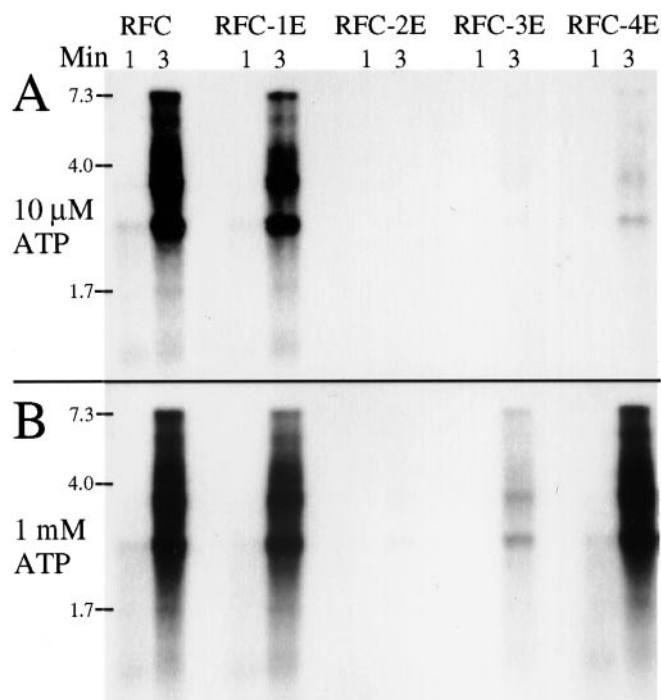


FIG. 7. Activity of RFC Lys → Glu mutants in primed mp18 DNA replication. DNA replication reactions at 30 °C containing 0.2 pmol of wild type or mutant RFC at 10  $\mu$ M ATP (A) or 1 mM ATP (B)

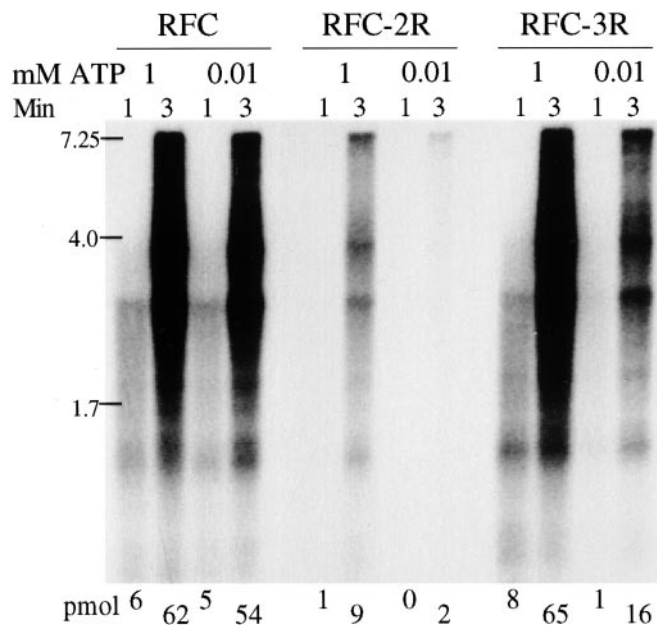


FIG. 8. Activity of RFC Lys → Arg mutants in primed mp18 DNA replication. DNA replication reactions at 30 °C containing 0.2 pmol of wild type or mutant RFC at the indicated levels of ATP. Total DNA synthesis (pmol of dNTP) is indicated below each lane.

mutant protein is disrupted but rather because an essential domain of the mutant protein is misfolded or because of improper interactions of the mutant protein with other subunits in the complex. We think that it is unlikely that gross misfolding or misassociation occurred in any of the mutant yeast complexes because all complexes efficiently interacted with PCNA in the presence of ATP or ATP $\gamma$ S, which represents a very specific step in the loading pathway (Fig. 3). In fact, the PCNA affinity column step that we applied to the purification of all mutant complexes not only served to remove excess Rfc2–5 core complex but also to remove misfolded, misassoci-

TABLE II  
Comparison between human and yeast RFC mutants  
The data for the human K → A mutants are from Ref. 9, and those for K → E are from Ref. 10. Minor, >30% activity; moderate, 5–30% activity; severe, 1–5% activity; inactive, <1% activity;  $K_m$ , no activity at 10  $\mu$ M ATP.

Yeast RFC			Human RFC		
Subunit	Mutation	Defect	Subunit	Mutation	Defect
Rfc1	Lys → Glu	minor	p140	Lys → Ala	severe
				Lys → Glu	inactive
Rfc2	Lys → Arg	moderate, $K_m$	p37	Lys → Ala	moderate
	Lys → Glu	inactive		Lys → Glu	severe
Rfc3	Lys → Arg	minor, $K_m$	p36	Lys → Ala	moderate
	Lys → Glu	severe, $K_m$		Lys → Glu	severe
Rfc4	Lys → Glu	minor, $K_m$	p40	Lys → Ala	severe
				Lys → Glu	inactive

ated, and partially proteolyzed inactive five-subunit complexes. For this reason, the yield of the RFC1-E complex after PCNA-agarose was extremely low.

The diametrically different results obtained with the yeast and human mutant Rfc1 complexes may reflect distinct mechanistic differences between human and yeast RFC, *i.e.* that ATP binding to human Rfc1, but not to yeast Rfc1, is essential for clamp loading. Another explanation is based on the assumption that yeast and human RFC are mechanistically similar but that serious problems occurred with the folding, stability, and/or subunit-subunit interactions in the human RFC complex containing mutant Rfc1. PCNA affinity chromatography was not included as a purification step of the human RFC preparations, and therefore it may well be possible that the preparations contained large amounts of misfolded or misassociated inactive complexes (9, 10). Indeed, in their study, Podust *et al.* (10) commented on the very low yield of RFC containing a Rfc1-K657E mutation from a baculovirus expression system.

**Binding of ATP to Rfc4 Is Required for Clamp Loading—**Without PCNA present, the kinetic parameters of the RFC-4E ATPase were independent of DNA, reflecting the inability of the mutant complex to bind DNA at low ATP concentrations (Fig. 4C and Table I). However, in the presence of PCNA, the stimulation of the RFC-4E ATPase by DNA is indicative of clamp loading, even though the kinetic parameters were negatively affected in comparison with wild type. The data were fitted to a Michaelis-Menten binding curve with a single  $K_m$  value of 40  $\mu$ M ATP (Table I), but an equally good fit was obtained with two different  $K_m$  values, *i.e.*  $K_{m1} = 2 \mu$ M, reflecting the PCNA-dependent ATPase at low ATP values where no DNA binding occurred, and  $K_{m2} = 70 \mu$ M, reflecting the PCNA and DNA-dependent ATPase. The latter value is similar to that obtained in the loading assays with ATP $\gamma$ S monitored by SPR (Fig. 4) and to the functional clamp loading assay on poly(dA)-oligo(dT) (Fig. 6).

**Subunit Redundancy in Binding of RFC to PCNA but Not to DNA—**One of the most surprising results of this study was that none of the mutations severely affected PCNA binding (Fig. 3). Of the three mutant complexes examined by SPR, none showed a substantial  $K_D$  effect for binding PCNA. Although similar studies were not carried out with RFC-1E, the ability of RFC-1E to bind PCNA-agarose at high salt concentrations in the presence of ATP as well as the wild type clamp loading properties of this mutant suggest that it should also bind PCNA similarly to wild type (Fig. 6) (14). These data suggest that at least two Rfc subunits with two ATPs bound are involved in binding PCNA and opening it in preparation for loading around the DNA. ATP occupancy of either one of these two subunits would suffice for the formation of a strong salt-resistant RFC-PCNA complex.

In contrast, stable DNA binding by RFC requires ATP occu-

pancy to Rfc2, Rfc3, and to Rfc4. Curiously, residual DNA binding was observed by RFC-2E and RFC-3E, but this binding was suppressed in the presence of PCNA. Previously, we have established an ordered mechanism for clamp loading in which binding of PCNA to RFC precedes binding of DNA (4). When the binding pathway proceeds via PCNA to DNA, the mutations in Rfc2E and Rfc3E cause a virtually complete block, and the mutation in Rfc4E causes an ATP concentration-dependent block in DNA binding. Possibly, this block is at a step related to PCNA opening, prohibiting the PCNA-RFC complex to go through the conformational change necessary for DNA binding.

In conclusion, this mutational study has shown that ATP binding to Rfc2, Rfc3, and Rfc4 is essential for clamp loading. Unexpectedly, we found that the ATP-binding domains of the Rfc subunits tolerate mutations remarkably well. As a result our analysis shows a gradient of phenotypes, indicating that mutations in each subunit differentially affect the ability to bind and hydrolyze ATP by the mutant subunit. Most severely affected is the complex with the Lys → Glu mutation in Rfc2, whereas a Lys → Glu mutation in Rfc4 shows only a quantitative defect in ATP binding but not in the ability to hydrolyze the bound ATP. The Lys → Arg mutation in Rfc3 has only marginal defects. Our *in vivo* studies of these mutants, presented in the fourth paper of this series, broadly confirm the results drawn from this biochemical study (31).

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## REFERENCES

- Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. (1995) *Mol. Cell. Biol.* **15**, 4661–4671
- Guenther, B., Onrust, R., Sali, A., O'Donnell, M., and Kuriyan, J. (1997) *Cell* **91**, 335–345
- Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) *Genome Res.* **9**, 27–43
- Gomes, X. V., Gary Schmidt, S. L., and Burgers, P. M. (2000) *J. Biol. Chem.* **276**, 34776–34783
- Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J.* **1**, 945–951
- Parsonage, D., Al-Shawi, M. K., and Senior, A. (1988) *J. Biol. Chem.* **263**, 4740–4744
- Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 907–911
- Green, C. M., Erdjument-Bromage, H., Tempst, P., and Lowndes, N. F. (2000) *Curr. Biol.* **10**, 39–42
- Cai, J., Yao, N., Gibbs, E., Finkelstein, J., Phillips, B., O'Donnell, M., and Hurwitz, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11607–11612
- Podust, V. N., Tiwari, N., Ott, R., and Fanning, E. (1998) *J. Biol. Chem.* **273**, 12935–12942
- Gomes, X. V., Gary, S. L., and Burgers, P. M. (2000) *J. Biol. Chem.* **275**, 14541–14549
- Li, X., and Burgers, P. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 868–872
- Li, X., and Burgers, P. M. (1994) *J. Biol. Chem.* **269**, 21880–21884
- Gerik, K. J., Gary, S. L., and Burgers, P. M. (1997) *J. Biol. Chem.* **272**, 1256–1262
- Gomes, X. V., and Burgers, P. M. (2000) *J. Biol. Chem.* **276**, 34768–34775
- Uhlmann, F., Cai, J., Gibbs, E., O'Donnell, M., and Hurwitz, J. (1997) *J. Biol. Chem.* **272**, 10058–10064
- Podust, V. N., Tiwari, N., Stephan, S., and Fanning, E. (1998) *J. Biol. Chem.* **273**, 31992–31999
- Tan, C. K., Castillo, C., So, A. G., and Downey, K. M. (1986) *J. Biol. Chem.* **261**, 12310–12316
- Bauer, G. A., and Burgers, P. M. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7506–7510
- Burgers, P. M. J., and Yoder, B. L. (1993) *J. Biol. Chem.* **268**, 19923–19936
- Podust, L. M., Podust, V. N., Floth, C., and Hubscher, U. (1994) *Nucleic Acids Res.* **22**, 2970–2975
- Li, X., Li, J., Harrington, J., Lieber, M. R., and Burgers, P. M. (1995) *J. Biol. Chem.* **270**, 22109–22112
- O'Brien, M. C., Flaherty, K. M., and McKay, D. B. (1996) *J. Biol. Chem.* **271**, 15874–15878
- Yang, g., Sandalova, T., Lohman, K., Lindqvist, Y., and Rendina, A. R. (1997) *Biochemistry* **36**, 4751–4760
- Smith, C. V., and Maxwell, A. (1998) *Biochemistry* **37**, 9658–9667
- Hishida, T., Iwasaki, H., Yagi, T., and Shinagawa, H. (1999) *J. Biol. Chem.* **274**, 25335–25342
- Mizushima, T., Takaki, T., Kubota, T., Tsuchiya, T., Miki, T., Katayama, T., and Sekimizu, K. (1998) *J. Biol. Chem.* **273**, 20847–20851
- Xiao, H., Naktinis, V., and O'Donnell, M. (1995) *J. Biol. Chem.* **270**, 13378–13383
- Mizushima, T., Takahashi, N., and Stillman, B. (2000) *Genes Dev.* **14**, 1631–1641
- You, Z. Y., Komamura, Y., and Ishimi, Y. (1999) *Mol. Cell. Biol.* **19**, 8003–8015
- Gary Schmidt, S. L., Pautz, A. L., and Burgers, P. M. J. (2001) *J. Biol. Chem.* **276**, 34792–34800