

ATP Utilization by Yeast Replication Factor C

I. ATP-MEDIATED INTERACTION WITH DNA AND WITH PROLIFERATING CELL NUCLEAR ANTIGEN*

Received for publication, December 22, 2000, and in revised form, May 3, 2001
Published, JBC Papers in Press, June 29, 2001, DOI 10.1074/jbc.M011631200

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Eukaryotic replication factor C is the heteropentameric complex that loads the replication clamp proliferating cell nuclear antigen (PCNA) onto primed DNA. In this study we used a derivative, designated RFC, with a N-terminal truncation of the Rfc1 subunit removing a DNA-binding domain not required for clamp loading. Interactions of yeast RFC with PCNA and DNA were studied by surface plasmon resonance. Binding of RFC to PCNA was stimulated by either adenosine (3-thiotriphosphate) (ATP γ S) or ATP. RFC bound only to primer-template DNA coated with the single-stranded DNA-binding protein RPA if ATP γ S was also present. Binding occurred without dissociation of RPA. ATP did not stimulate binding of RFC to DNA, suggesting that hydrolysis of ATP dissociated DNA-bound RFC. However, when RFC and PCNA together were flowed across the DNA chip in the presence of ATP, a signal was observed suggesting loading of PCNA by RFC. With ATP γ S present instead of ATP, long-lived response signals were observed indicative of loading complexes arrested on the DNA. A primer with a 3' single-stranded extension also allowed loading of PCNA; yet turnover of the reaction intermediates was dramatically slowed down. Filter binding experiments and analysis of proteins bound to DNA-magnetic beads confirmed the conclusions drawn from the surface plasmon resonance studies.

The elongation apparatus for DNA replication is functionally conserved in all free living organisms, and a similar apparatus has been found in some bacteriophages, *e.g.* T4 (see Ref. 1 for a review). Processive DNA replication by the replicative DNA polymerase depends on its interaction with a toroidal shaped protein, the replication clamp. This clamp is loaded onto the template-primer junction by a protein complex, the clamp loader. Replication factor C (RF-C),¹ the eukaryotic clamp loader, was first identified and purified as an essential component for SV40 DNA replication (2). It is a multipolypeptide complex that loads the replication clamp proliferating cell nuclear antigen (PCNA) onto the template-primer junction in

an ATP-dependent manner. PCNA is the processivity factor for several eukaryotic DNA polymerases including DNA polymerase δ .

Yeast RF-C consists of a large subunit with a molecular mass of 95 kDa and four smaller subunits of 36–40 kDa. The genes encoding all five subunits are essential (3–8). All five subunits show sequence similarity to each other and to Rfc subunits from eukaryotes in general. This homology is localized in seven regions known as RF-C boxes II–VIII (reviewed in Ref. 7). RF-C boxes III and V contain sequences that show homology to nucleotide-binding proteins (9). *RF1* contains an additional box (I) in the N-terminal region that shows homology to prokaryotic DNA ligases and poly(ADP)-ribose polymerases (10). This box is not required for the clamp loading function of RF-C (11, 12), and in fact, deletion of the N-terminal domain containing box I from yeast *RF1* shows no detectable replication phenotype and only a marginal repair phenotype (13). The C termini of all five subunits are unique and are required for complex formation (11, 14).

Biochemical studies of the eukaryotic clamp loader RF-C have established that the complex has a preferential binding affinity for template-primer junctions and has a single-stranded DNA-stimulated ATPase activity that is further activated by the presence of primer termini and PCNA (15–19). In the presence of ATP or ATP γ S a strong complex is formed between RF-C and PCNA, and in the presence of ATP γ S a strong complex is formed between RF-C and DNA (16, 20). Hydrolysis of ATP γ S is not observed, indicating that binding rather than hydrolysis of ATP drives formation of these two distinct complexes.

Comprehensive mechanistic studies of the role of ATP in clamp loader interactions with the clamp and with DNA have been documented for the T4 and the *Escherichia coli* system (21–24). Given the structural similarities of the three model systems, one might expect that the mechanism of clamp loading in eukaryotes would be similar in detail to T4 and *E. coli*. However, there are at least two reasons why there may be substantial differences. First, all subunits except *RF5* have a consensus ATP-binding domain, suggesting the possible involvement of four ATPs in the reaction pathway. However, unlike the T4 clamp loader, these putative four ATP molecules would be localized in unique rather than identical subunits, likely assigning an unique function to each subunit and the ATP bound to it. Second, there is accumulating evidence that the four small subunits constitute a core complex, designated Rfc2–5, that can associate with any subunit of a family of large subunits to form different complexes with distinct functions: with Rfc1p to form RF-C; with Rad24p to form Rad24Rfc2–5, which functions in checkpoint control; and with Chl12p to form Chl12Rfc2–5, which may function in a termination step in DNA replication (25, 26). Therefore, it is well possible that the eu-

* This work was supported in part by Grant GM32431 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: RF-C, replication factor C; RFC, replication factor C with Rfc1- Δ (3–272); Rfc2–5, complex of Rfc2p, Rfc3p, Rfc4p, and Rfc5p; RPA, replication protein A; PCNA, proliferating cell nuclear antigen; SS, single-stranded; ATP γ S, adenosine (3-thiotriphosphate); SPR, surface plasmon resonance; RU, resonance units.

karyotic clamp loader has evolved a modular binding and usage of ATP molecules that allow a more flexible adaptation for function in these different types of clamp loaders. In addition, specific functions may be associated with the Rfc2–5 core complex to guide its assembly into appropriate clamp loader complexes.

In this series of papers we present studies detailing the mechanism of PCNA loading by RF-C and the requirement of ATP in this process. RF-C has at least two types of DNA-binding domains. DNA binding by a domain localized in the N-terminal third of Rfc1p is ATP-independent and unrelated to clamp loading. The DNA-binding sites in the C-terminal domain of Rfc1p and in other subunits of the complex may function as a coordinate unit that requires ATP for interaction with DNA. To facilitate our studies of the role of ATP in DNA binding and clamp loading, we have used a truncation derivative of RF-C in which the N-terminal domain carrying the ATP-independent DNA-binding domain has been deleted. Like the analogously truncated human RF-C, this derivative complex has an increased clamp loading activity that can be attributed to the loss of a competing DNA-binding domain for non-primer-template junctions (11–13). The truncation derivative of RF-C containing Rfc1p Δ (3–272) has been used in all of our biochemical studies in these papers, and, for ease of reading, this complex has been simply designated as RFC.

This paper details the ATP-dependent interactions of RFC and the Rfc2–5 core with DNA and PCNA and the effect of the single-stranded DNA-binding protein RPA and mismatched primer termini on binding and loading. These studies use surface plasmon resonance (SPR) for measuring interactions, but additional techniques have been presented to validate the SPR approach for studying clamp loading. The second paper details the quantitative aspects of ATP in the formation of complexes, clamp loading, and the order of the reaction pathway (27). The third paper reports biochemical studies of mutant RFC complexes with mutations in the ATP-binding domains of four out of the five subunits (28). A complementary genetic study of these mutants is reported in the fourth paper of this series (45).

EXPERIMENTAL PROCEDURES

Enzymes, DNA, and Buffers—PCNA, Rfc2–5, and replication protein A (RPA) were purified from *E. coli* overproduction strains as described (13, 29, 30). A truncated form of RF-C, in which residues 3–272 from Rfc1p was deleted, was used in this study (13). The concentrations of RFC and PCNA were determined spectrophotometrically in 7 M guanidinium hydrochloride using the calculated extinction coefficient from the protein sequences. All other enzymes and oligonucleotides were obtained commercially. ATP γ S was obtained from Roche Molecular Biochemicals. Buffer A contained 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 P40. Buffer B contained buffer A with 0.2 mg/ml bovine serum albumin.

Filter Binding—Whatman nitrocellulose filters (0.2 μ m) were treated for 45 min with 0.4 M KOH and then equilibrated in buffer A. The V6 oligonucleotide (see Fig. 1A) was 5'-end labeled with 32 P and hybridized to primer C12 in a V6:C12 molar ratio of 1:1.5. The DNA (0.65 nM) was incubated with 0.375–16 nM RFC in 30 μ l of buffer B with 100 μ M ATP γ S for 5 min at 0 °C and then filtered through the nitrocellulose filter. The filter was washed with 0.5 ml of buffer A and dried, and the radioactivity was counted in a scintillation counter.

ATPase Assays—10- μ l assays were performed in buffer B, except that the final NaCl concentration was 75 mM. The assays contained 0.1 μ M Rfc2–5 or RFC, 50 μ M [α - 32 P]ATP, 0.5 μ M *E. coli* single-stranded binding protein, and when present 0.5 μ M PCNA and 1 μ M V6 or 1 μ M V6/C12 DNA (see Fig. 1A). After 6 min at 30 °C, the reaction was quenched with 3 μ l of 50 mM EDTA, 1% SDS, 20 mM each of ADP and ATP. 3 μ l was spotted on a polyethyleneimine cellulose sheet and dried. The sheet was washed in distilled water for 10 min, rinsed in ethanol, dried, and developed in 0.5 M LiCl, 1 M HCOOH. The sheets were dried and subjected to PhosphorImager analysis (Molecular Dynamics).

Bi-molecular Interaction Analysis—SPR was performed in a BIAcore

X apparatus. Buffer B was the running buffer used in the analysis. When a DNA chip was used, ~2000 resonance units (RU) of streptavidin were immobilized on the surface of a dextran chip (pioneer F1) by carbodiimide coupling according to the manufacturer's instructions. A biotinylated 80-mer template (see Fig. 1A), either alone or hybridized to an excess of primer C12 (or C12T, C12T3, or C12T10), was attached to the chip via the streptavidin-biotin linkage. Approximately 20–30 RU of template were immobilized. When a PCNA chip was used, ~30–100 RU of PCNA were covalently immobilized on the surface of the dextran chip (CM5) by a carbodiimide-activated succinimide coupling method (amine coupling) according to the manufacturer's instructions. This mild coupling chemistry is analogous to one we previously used for the coupling of PCNA to agarose beads that proceeded largely with retention of RFC binding activity (20).

The interaction between RFC with PCNA and with DNA was monitored at 20 °C by injecting 90 μ l of the indicated concentrations of RFC over a PCNA chip or DNA chip at a flow rate of 30 μ l/min. Higher flow rates did not significantly increase the rate of RFC binding to the chips, indicating that surface effects do not pose a serious problem with these low density chips. The dissociation constants (K_D) were calculated using software provided by the manufacturer. Each K_D value was obtained from in general 7–10 injections. Interaction measurements with the Rfc2–5 core were done similarly.

Isolation of Complexes of PCNA and RFC on DNA Bound to Magnetic Beads—The biotinylated 80-mer template V6 was hybridized to the 30-mer primer C12 (see Fig. 1A) to generate the matched substrate or hybridized with C12T3 (5'-C12TTT) to generate the mismatched substrate. The primer-template substrate was immobilized onto streptavidin magnetic beads (Dynabeads) in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 M NaCl by incubating at room temperature for 1–2 h. All washes were carried out using a Dynal magnet with a volume of buffer 100–200 times the bead volume for 1–2 min each at room temperature. The unbound substrate was washed off the beads twice with buffer B. The binding assay was performed in a 20- μ l reaction in buffer B. About 500 fmol of DNA bead substrate (1 μ l of beads) was coated with 5 pmol of yeast RPA for 1 min followed, where indicated, by the addition of 3 pmol of PCNA, 100 μ M ATP γ S, or 1 mM ATP and the indicated amounts of RFC. The reaction was incubated at 30 °C for 1 min. The beads were washed three times with wash buffer B. The same number of washes were carried out for each experiment regardless whether specific components were left out of the assay. Bead-bound proteins were boiled in sample loading buffer and separated on a SDS-10% polyacrylamide gel. The proteins were blotted onto a nitrocellulose membrane using a Mini Trans-Blot electrophoretic transfer cell from Bio-Rad. The blot was probed with a mixture of polyclonal antibodies raised in rabbit against PCNA and Rfc3p. Detection was carried out using an ECL chemiluminescence kit (Amersham Pharmacia Biotech) as recommended by the manufacturer. Different exposures of the blot were photographed with a CCD camera and digitized for quantitation.

RESULTS

Surface Plasmon Resonance for Studying Clamp Loading—Previously, we had shown that RFC, *i.e.* RF-C lacking the ligase homology domain, is fully competent for clamp loading (13). The ligase homology domain localizes to the N-terminal domain of Rfc1p and binds DNA regardless of the presence of ATP (31, 32). Removal of the ligase homology domain revealed that binding of the remaining complex, *i.e.* RFC, to DNA was strongly stimulated by ATP γ S, a nonhydrolyzable analog of ATP (13). These results are in agreement with earlier footprinting studies of human RF-C to DNA and recent binding studies with the *E. coli* γ -complex (16, 33).

To obtain a quantitative understanding of these interactions, we have used SPR. This technique, in which protein or DNA is attached to a dextran-coated surface and a second component is flowed across this chip to detect binding and dissociation in one experiment, may sometimes yield incorrect quantitative information because of surface effects (34). In general these effects are minimized or eliminated if the surface of the chip is charged to a very low density with the immobilized component and if the flow rate across the chip is high enough so that rapid exchange between the surface-proximal and surface-distal fluid layers occurs. To determine whether SPR would be a useful technique providing accurate and meaningful quantitative in-

FIG. 1. Quantitative measurement of RFC-DNA interactions. A, the 80-mer template V6 (5'-T₃₀CTCCCTTCCTCCTCCCTTCCCTT₂₁-Biotin) was ³²P-labeled at the 5'-end and then hybridized to the 30-mer primer C12 (5'-AGGGAAGGGAGAGGGAGGAGAAGAA-GGGAG) for the filter binding experiment in B or hybridized to the 30-mer primer and then attached via the biotin linkage to a BIAcore P-streptavidin chip for the SPR experiment in C. The binding experiments in B and C were carried out at increasing RFC concentrations and 100 μM ATP_γS.

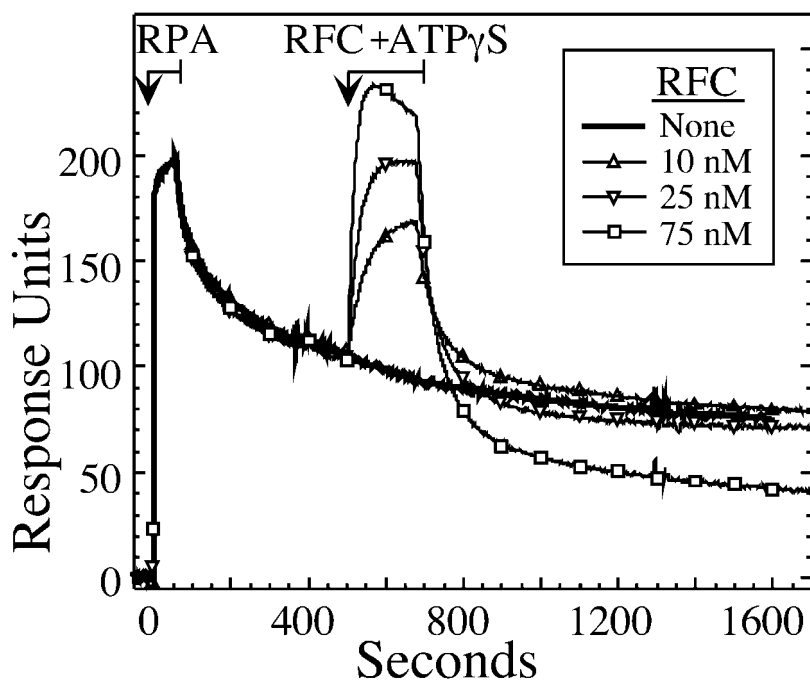
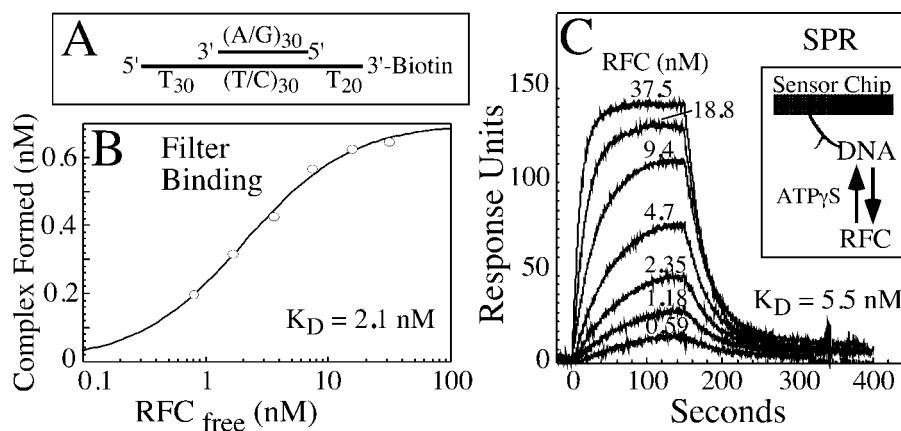


FIG. 2. Concurrent binding of RPA and RFC to DNA. RPA (40 nM) was injected onto the DNA chip (see Fig. 1A) from $t = 0$ s to $t = 45$ s. From $t = 500$ s to $t = 680$ s, either buffer containing 100 μM of ATP_γS (*None*) or the indicated concentrations of RFC in the same buffer were injected.

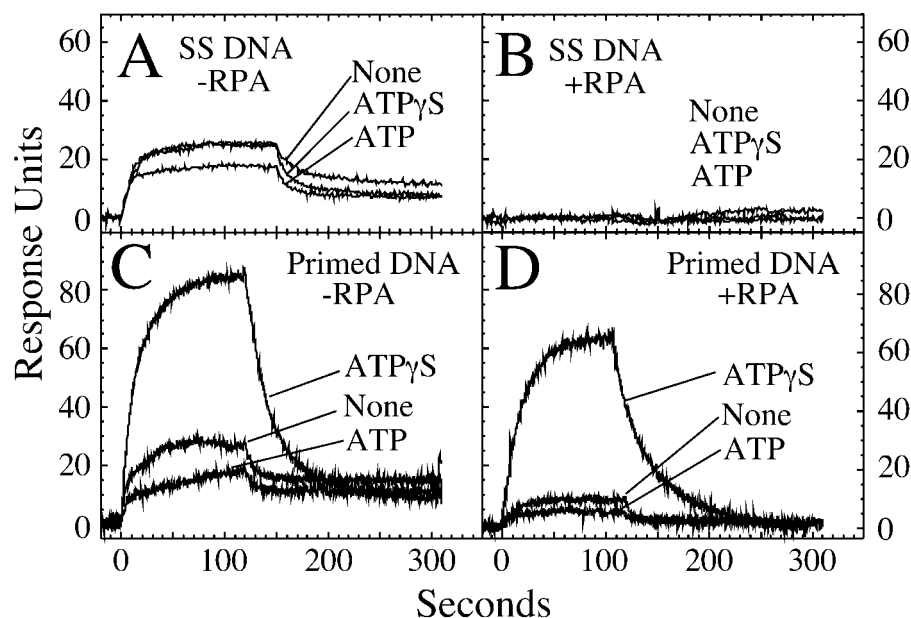
formation for interactions between DNA and RFC, we attached a 30/80-mer primer-template to the sensor chip via a biotin-streptavidin linkage (Fig. 1A). The amount of DNA bound to the chip was kept very low at ~20 RU to avoid problems inherent to high density chips (the maximum binding capacity of the chip is >1000 RU).

Binding of RFC to the DNA chip in the presence of a saturating concentration of ATP_γS (100 μM) was measured at increasing RFC concentrations (Fig. 1C). Upon injection of RFC, rapid binding to the DNA chip was observed until an equilibrium, the steady state response, was reached. The observed k_{on} values ($3-4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and k_{off} values ($0.022-0.028 \text{ s}^{-1}$) were largely independent on the RFC concentration between 1.2 and 75 nM, and a global fit of the data yielded a K_D value of 6 ± 2 nM RFC. A plot of the steady state binding levels against RFC concentration gave a K_D value of 5.5 ± 2 nM for the RFC-DNA interaction. In comparison, classical filter binding experiments under the exact same solution conditions with the same template-primer, but now labeled with ³²P at the 5'-end of the 80-mer, gave a K_D value of 2.1 ± 1 nM (Fig. 1B). We consider the K_D values obtained by these two techniques sufficiently close to conclude that SPR can provide reliable quantitative information about protein-protein and protein-DNA interactions.

The DNA substrate used in this study is a partial duplex in which the A/G-rich primer strand is hybridized to a C/T-rich template strand and the SS regions of the template strand consist of oligo(dT) (Fig. 1A). The choice of this set eliminated the potential for secondary structure and allowed stable binding of RPA to the SS DNA (see below) (35). When a natural DNA template was used, derived from M13-mp18 sequences, the half-life of bound RPA was unacceptably low at 10–15 min. Furthermore, because of primer dimer and secondary structure formation, little or no dependence on a primer-template junction for binding by RFC was observed (data not shown).

Binding of RFC to RPA-coated Primed DNA—To evaluate whether SPR would permit detection of complexes containing both RPA and RFC, the DNA chip was first injected with RPA, followed by injection of RFC. Separate experiments showed that saturation binding of RPA was achieved with 40 nM RPA (data not shown). After injection of RPA stopped, an initial rapid dissociation of a minor fraction of weakly bound RPA was observed, followed by a very slow dissociation of the remaining RPA ($t_{1/2} = 50$ min) (Fig. 2). When during this slow dissociation phase 10 nM RFC in ATP_γS buffer was injected, a sharp increase in signal was observed, followed by a subsequent decrease in signal when the injection of RFC was switched to that of buffer, suggesting that a binding of RFC was observed sim-

FIG. 3. RPA provides specificity of binding to primer-template DNA. At $t = -450$ s, 40 nM RPA was injected onto DNA chip, and the interaction with DNA was monitored. At $t = 0$, 25 nM RFC was injected in buffer containing no nucleotides (*None*) or in the presence of 1 mM ATP or 100 μ M ATP γ S. Shown is a sensorgram of RFC binding to unprimed 80-mer template in the absence of RPA (A) or in the presence of RPA (B) or binding of RFC to primed 80-mer template in the absence of RPA (C) or in the presence of RPA (D).



ilar to that in Fig. 1C. Remarkably, after several minutes of dissociation, the response curve closely matched that of the control curve in which buffer was injected instead of RFC (Fig. 2, *None*). The same results were obtained with 25 nM RFC. This suggests that binding of RFC did not displace the bound RPA. Because an equilibrium was reached during the injection period when 10 or 25 nM RFC were injected, it is likely that the available primer-templates were multiple times sampled by RFC binding. In addition, because the site size of RPA is ~ 30 nucleotides, only two RPA molecules would be expected to bind to the DNA, one on each side of the primer (35). Therefore, dissociation of one RPA molecule upon binding of RFC would result in a 50% decrease in the magnitude of the slow decay signal after RFC dissociation was complete, *i.e.* after ~ 1000 s. This was not observed, indicating that RPA and RFC can bind the DNA substrate concurrently. Finally, when very high concentrations of RFC were injected on the RPA-coated DNA chip, some dissociation of the prebound RPA was observed. This was suggested by a decrease in the response signal during the binding phase after the initial maximum response had been reached (at 600–700 s) and, secondly, by the occurrence of a residual signal significantly lower than the control during the latter part of the dissociation phase (Fig. 2). Therefore, most of our DNA binding studies with RPA-coated DNA chips were carried out at 10–25 nM RFC.

RPA Provides Specificity of Binding to Primer-template DNA—Although the 80-mer DNA template used in these studies did not contain any obvious sequences that could form secondary structures (Fig. 1A), low but significant RFC binding was observed to a chip to which only the SS template oligonucleotide was attached (Fig. 3A). However, this binding was independent of a nucleotide cofactor, indicative of its nonspecific nature. Moreover, coating of the SS DNA with RPA prior to injection of RFC eliminated all binding of RFC (Fig. 3B). In contrast, when primer-template was attached to the chip, robust binding of RFC was observed in the presence of ATP γ S, and weak binding was observed when ATP or no nucleotide was present (Fig. 3C). Interestingly, the level of binding in the presence of ATP was consistently and significantly lower than in its absence, suggesting that hydrolysis of ATP may actively promote dissociation of DNA-bound RFC. Coating of the primed DNA with RPA prior to injection of RFC virtually eliminated binding with or without ATP, whereas binding with

ATP γ S showed only a slight reduction (Fig. 3D). The simplest explanation for these results is that binding of RFC to primed DNA requires binding of ATP but that hydrolysis of the bound ATP promotes complex dissociation. These studies assume that the observed differences between experiments with ATP and with ATP γ S derive not from differences in binding affinities between these nucleotides to RFC but rather from the inability of ATP γ S to undergo hydrolysis. Control studies established that ATP γ S is not hydrolyzed by RFC under any binding condition, *i.e.* with or without PCNA and/or DNA (data not shown).

The affinity of the Rfc2–5 core for primed DNA was extremely low and could not be reliably measured (data not shown and Table I). Furthermore, this low binding was unaffected by the presence of RPA or a nucleotide cofactor. When the NaCl concentration in the buffer used for SPR experiments was decreased from 125 to 75 mM, still no specific interaction between Rfc2–5 and DNA was observed, but nonspecific binding of Rfc2–5 to the chip matrix increased (data not shown). Despite these negative results, ATPase data presented below show indirectly that the core complex interacts with DNA albeit weakly.

Interaction of RFC and Rfc2–5 with PCNA—Previously, we have shown that ATP or ATP γ S greatly enhances the binding of RFC to PCNA-agarose beads (20). PCNA was attached at a very low density (~ 60 response units) to a sensor chip using activated amine chemistry. A weak interaction with RFC was detected under the same solution conditions used for detection of RFC-DNA complexes (see “Experimental Procedures” and Fig. 4A). However, inclusion of ATP or ATP γ S during the injection increased the affinity over 10-fold (Fig. 4A and Table I).

The binding data with the Rfc2–5 core to the PCNA chip contrast remarkably with those for RFC. Very low but significant binding with an estimated K_D of ~ 500 nM was observed for Rfc2–5 to the PCNA chip. The addition of ATP γ S increased the affinity over 10-fold (Fig. 4B). However, no increased binding was observed in the presence of ATP, suggesting that hydrolysis of ATP released the PCNA-bound Rfc2–5.

ATPase Activity of RFC and Rfc2–5—In the absence of DNA, both RFC and Rfc2–5 showed a weak ATPase activity, which was stimulated 3–5-fold by PCNA (Fig. 5). As shown before, the ATPase of yeast RFC was moderately stimulated by SS DNA and substantially by primed DNA, and a synergistic increase

TABLE I
Interaction of RFC and Rfc2-5 with PCNA and DNA

Increasing concentrations of RFC or Rfc2-5 were flowed across the sensor chip, and the K_D values were determined from a plot of the steady state response against protein concentration (Fig. 1C). The K_{off} values were determined separately and for all experiments were independent of the concentration of RFC. All binding studies to the DNA chips were performed in the presence of RPA. No Binding indicates that the estimated K_D is > 1000 nM.

Sensor Chip	Analytes				K_D	k_{off}
	RFC	PCNA	ATP	ATP γ S		
PCNA	+	-	-	-	21	0.035
	+	-	+	-	1.3	0.011
	+	-	-	+	1.5	0.011
	Rfc2-5	-	-	-	~500	
	Rfc2-5	-	-	+	~500	
Matched primer-template	Rfc2-5	-	-	+	40	
	+	-	-	-	No Binding	
	+	-	+	-	No Binding	
	+	-	-	+	15	0.013
	+	+	-	-	No Binding	
	+	+	+	-	Binding ^a	0.09, 0.007 ^b
	+	+	-	+	5	0.007
	-	+	-	+	No Binding	
Rfc2-5	-	-	+	No Binding		
Rfc2-5	+	-	-	+	No Binding	
Forked primer template	+	-	-	+	10	0.013
	+	+	+	-	Binding ^a	0.02, 0.003 ^b
	+	+	-	+	1.3	0.003
	+	+	-	+		

^a K_D cannot be calculated because of bi-modal dissociation.

^b k_{off} rates for dissociation of RFC and PCNA, respectively.

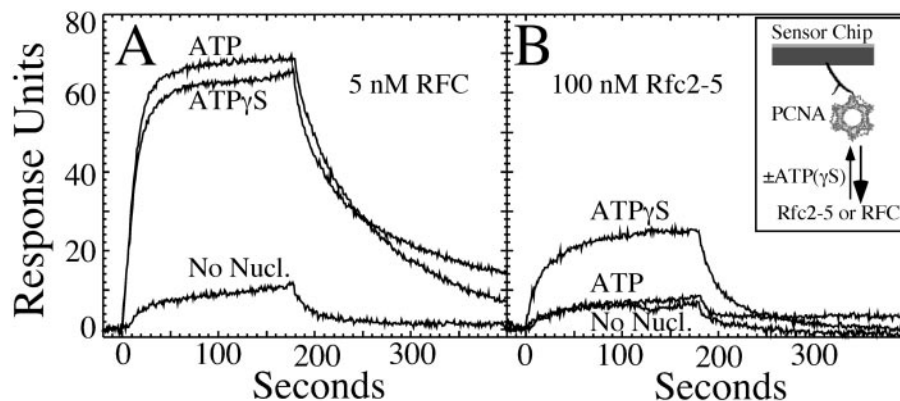


FIG. 4. **ATP-dependent binding of RFC or Rfc2-5 to PCNA.** Either 5 nM RFC (A) or 100 nM Rfc2-5 (B) was injected over a PCNA chip in buffer containing no nucleotide, 1 mM ATP, or 100 μ M ATP γ S, as indicated.

was observed when both PCNA and primed DNA were present (18, 19). No significant stimulation of the ATPase of Rfc2-5 by SS DNA was observed, either with or without PCNA present. However, a 2-fold stimulation of the Rfc2-5 ATPase by primed DNA was observed, indicating indirectly that Rfc2-5 binds to primed but not to unprimed DNA. In the presence of both primed DNA and PCNA, the ATPase of Rfc2-5 was further enhanced but remained much lower than the activity observed with RFC (Fig. 5).

PCNA Loading on Primed DNA—As shown in previous studies, PCNA is not loaded onto SS DNA (16, 17, 36). In accordance, no response signal was observed when RFC together with a molar excess of PCNA, with or without ATP or ATP γ S, was injected onto a SS DNA chip previously coated with RPA (Fig. 6A). However, with primed DNA, three distinct response curves were observed depending on the nucleotide cofactor. In the absence of ATP or ATP γ S, no response signal was detected, indicating that PCNA was not loaded (Fig. 6B). In the presence of ATP, a robust signal was observed. As there was no detectable SPR signal when RFC and ATP without PCNA were flowed across the chip, the observed signal with RFC plus PCNA and ATP most likely reflects that of PCNA loading onto the DNA (compare Fig. 3D with Fig. 6B).

The dissociation curve in the presence of ATP consisted of two phases, an initial rapid phase (~ 0.09 s $^{-1}$), which we at-

tribute to dissociation of RFC, and a slower phase (~ 0.007 s $^{-1}$), which may represent dissociation of PCNA (Table I). Thus, the loaded PCNA was quite unstable and presumably dissociated from the DNA by sliding off the end (37). When ATP γ S was used as the cofactor, two significant differences were observed. First, the maximal steady state response was higher than the maximal response with ATP, and second, the dissociation of the bound complex with ATP γ S followed pseudo first-order kinetics with a single rate constant of 0.007 s $^{-1}$, indicating that the PCNA-RFC complex dissociated as a unit.

Increasing concentrations of RFC (from 5–50 nM) at a constant concentration of PCNA of 100 nM with either 1 mM ATP or 100 μ M ATP γ S present were injected onto the RPA-coated primed DNA chip. Maximal binding values were obtained from analysis of the response curves (Table I). The maximal response with ATP γ S present was 70% higher than that obtained with ATP. The most likely explanation for this difference is that PCNA is loaded onto the DNA, but with ATP γ S present RFC remains also bound. Thus, these results suggest that hydrolysis is required to complete the loading cycle and dissociate RFC.

To determine whether our conclusions from the SPR sensorgrams regarding the loading reaction with ATP or ATP γ S were correct, the same primer-template was attached to magnetic beads, and the loading reactions were repeated. RFC and PCNA bound to the beads were detected by a Western analysis.

The results were strikingly similar to those obtained with the SPR technique. With ATP γ S present in the loading reaction, both PCNA and RFC remained stably bound to the beads as had been inferred from the SPR experiments (Fig. 7B). However, with ATP in the loading reaction, no RFC and little PCNA was detected on the beads indicating that (i) RFC dissociates rapidly upon completion of loading and (ii) PCNA does not remain stably bound to this small oligonucleotide template-primer system (Fig. 7B). Similar problems with PCNA stability on small templates was observed in the human system (38). On the other hand, when the single-stranded region distal to the beads was extended to \sim 130 nucleotides, the loaded PCNA remained stably bound (39).

Loading of PCNA on Forked DNA Substrates—The effect of a non-base-paired primer terminus on the kinetics and efficiency of RFC-DNA interaction and on PCNA loading was investigated using SPR. For the experiment we used either a dT, a (dT) $_3$, or a (dT) $_{10}$ 3'-extension of the 30-mer primer. The primers were hybridized to the V6 template and attached to a chip. The results with the primer-template containing the one nucleotide 3'-extension, a T-T mismatch, were not significantly different from those observed with the fully base-paired DNA substrate (data not shown). Both the (dT) $_3$ and (dT) $_{10}$ forked DNA substrates showed binding and loading properties very similar to each other (data not shown) but distinctly different from those observed on the fully base-paired or single nucleotide mismatch substrate. PCNA loading on the (dT) $_3$ forked substrate was investigated in detail, and the results are shown in Figs. 6C and 7.

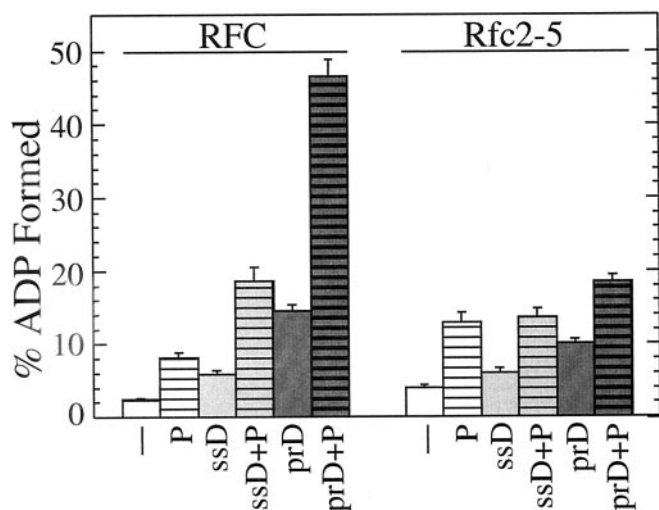
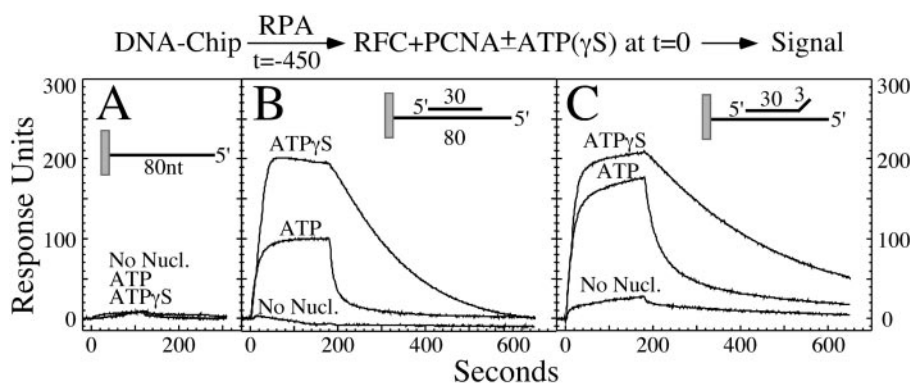


FIG. 5. ATPase activities of RFC and Rfc2-5. The assays were carried as described under "Experimental Procedures." The data are the averages of three independent experiments. P indicates PCNA. ssD indicates the V6 oligonucleotide, and prD indicates the C12/V6 primer-template. The ATP concentration was 50 μ M.



DISCUSSION

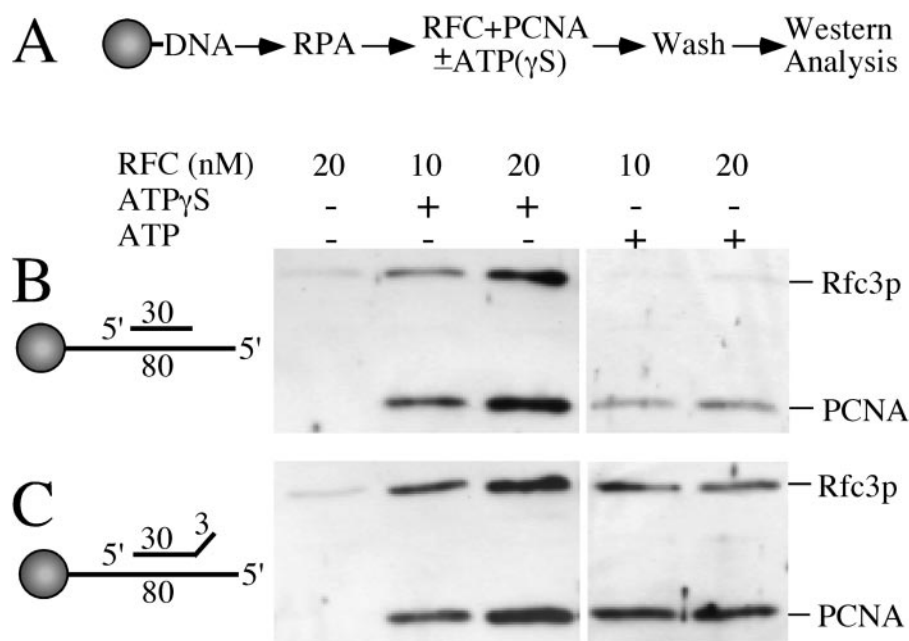
Loading of PCNA onto the forked substrate did appear to proceed efficiently. However, the maximal response with PCNA in the presence of ATP was similar to that observed in the presence of ATP γ S. Secondly, the dissociation rates of the complexes from the forked DNA were much lower than the analogous rates from the matched DNA (Table I). This suggests a failure of RFC to release efficiently from the DNA template after PCNA loading, even with ATP present. Alternatively, or in addition, more efficient retention of PCNA could also contribute to the increased signal. In support of the former explanation, the magnetic bead assay showed that in the presence of ATP, RFC remained bound to the forked DNA, whereas it had dissociated from the base-paired primer-template (Fig. 7C). One possible explanation for this result is that ATP hydrolysis did not occur on the forked DNA. However, the PCNA-stimulated ATPase activity of RFC on the forked DNA was indistinguishable from that on the fully base-paired primer-template DNA (Fig. 5 and data not shown), perhaps indicating that RFC might have a higher affinity for forked DNA structures. The observation that RFC shows some binding to forked DNA in the absence of a nucleotide cofactor supports this conclusion (Fig. 6C).

The results presented in this study put previous studies of the eukaryotic clamp loader in a quantitative frame work and extend our understanding of the process. The interaction of RFC with primer-template DNA is dependent on ATP binding but not ATP hydrolysis. In fact, ATP hydrolysis actively causes dissociation of RFC (Fig. 8). Consistent with this conclusion, RFC binding to either unprimed or primed DNA was reduced when ATP was present compared with no nucleotide (Fig. 3, A and C). Specificity of binding by RFC and RFC-PCNA to primer-template termini was achieved by coating of the DNA with RPA, in agreement with earlier studies of human RFC (16). Protein-protein interactions have been demonstrated between human RFC and RPA (40). However, coating of primer-template DNA with RPA did not increase the affinity of RFC for DNA. Rather it reduced the K_D value from 5 to 15 nM, suggesting that RPA, when bound to the DNA, did not promote RFC binding (compare Fig. 1B with Table I). On the other hand, no displacement of RPA was observed upon binding of RFC to the primer-template, indicating that concurrent binding of RFC and RPA occurred (Fig. 2).

The interactions of the Rfc2-5 core with primer-template DNA were extremely weak and could only be detected indirectly by the observation that primed but not unprimed DNA stimulated the ATPase activity of Rfc2-5 (Fig. 5). The apparent discrepancy between the SPR and ATPase studies can be rationalized considering that the DNA concentration in the ATPase assay (1 μ M primer-template junctions) vastly exceeded the DNA concentration on the chip. The human Rfc2-4

FIG. 6. PCNA loading onto matched and forked primer-templates. At $t = -450$ s, 40 nM RPA was injected onto a unprimed 80-mer template chip (A), a matched primer-template chip (B), or a forked primer-template chip (C), and the interaction with DNA was monitored. At $t = 0$, 25 nM RFC and 150 nM PCNA was injected in buffer containing no nucleotide, 1 mM ATP, or 100 μ M ATP γ S, as indicated. The mismatch primer C12T3 is 5'-AGGGAAGGGAGAGGGAGGAGAA-GAAGGGAGTTT (see also the legend to Fig. 1).

FIG. 7. **RFC remains bound to DNA when PCNA is loaded with ATP onto forked primer-templates.** A, outline of the assay. The DNA was attached to magnetic beads via a biotin-streptavidin linkage. The matched (B) or forked (C) primer-template was incubated in a stepwise fashion with the indicated proteins and nucleotide, and the beads were processed for immunoblot analysis. Anti-Rfc3p antibodies were used to detect RFC (see the legend to Fig. 6 and "Experimental Procedures" for details).



complex also showed preferential ATPase activity with primed *versus* unprimed DNA (41). The primer-template binding preference of Rfc2–5 minimally resides in the Rfc2 subunit, but DNA binding to yeast Rfc3 has also been inferred from its DNA-dependent ATPase activity (4, 5, 42). However, the Rfc3 ATPase does not show a preference for primer-template junctions. The very weak binding and poor stimulation of the Rfc2–5 ATPase by DNA contrasts with the readily detectable binding and DNA-stimulated ATPase of the individual subunits and is an indication that the DNA-binding domains of those subunits are (partially) buried inside the core and only become available during the loading process. For RFC this refers to loading of PCNA, but for other complexes containing the core, *e.g.* the Rad24Rfc2–5 complex, this may refer to loading of an alternative clamp (25).

In addition to a well characterized PCNA-binding domain in the Rfc1 subunit, PCNA binding has also been identified with the human Rfc3 and Rfc4 subunits (31, 42, 43). The human three-subunit Rfc2–4 complex also binds PCNA (41). Unexpectedly, we found that ATP γ S stimulated complex formation between Rfc2–5 and PCNA, whereas ATP did not (Fig. 4). Therefore, hydrolysis of ATP dissociated the PCNA-core complex. This is in contrast to the PCNA-RFC complex, which is maintained by either ATP or ATP γ S (Fig. 4). Yet, both RFC and Rfc2–5 showed comparable ATPase activities that were equivalently stimulated by PCNA (Fig. 5). Therefore, it appears that hydrolysis of bound ATP releases PCNA from the Rfc2–5 core, but the presence of the PCNA-binding domain in Rfc1 stabilizes PCNA onto RFC despite turnover of ATP. Perhaps, unlike Rfc2–5, the Rfc1 domain is not subject to allosteric control by ATP. The significance of this distinction may be of importance in the cell in that only RFC interacts productively with PCNA. The Rfc2–5 core, either alone or in complex with an alternative large subunit, binds PCNA poorly and actively dissociates bound PCNA to allow binding of putative alternative clamps.

Clamp loading on non-primer-template substrates has been studied recently in the *E. coli* system (44). The β clamp could be loaded on a wide variety of DNA substrates. However, the rates of loading and the fate of the clamp loader was not addressed in this study. Because 3'-SS tails are important for initiating recombination, we studied clamp loading on model substrates with 3'-SS tails of increasing length. Although a single mismatch did not affect the kinetics of clamp loading, surprisingly,

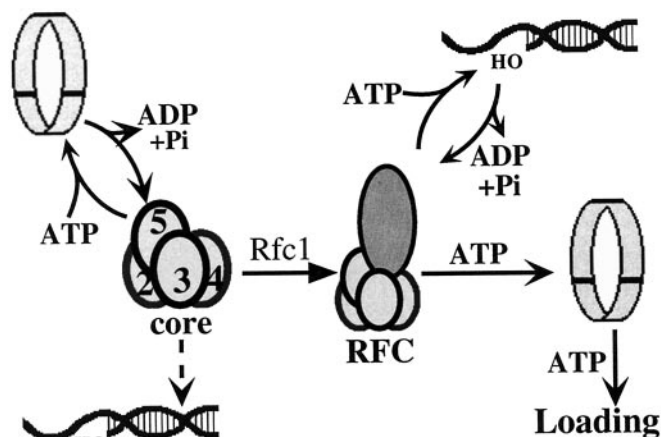


FIG. 8. **Model of the ATP-dependent interactions of RFC and Rfc2–5 with PCNA and with DNA.** The dashed arrow indicates a weak interaction.

release of RFC was inhibited when PCNA was loaded on forked substrates (Figs. 6C and 7C). It is unlikely that this inhibition is caused by a failure of ATP to hydrolyze upon completion of loading because the DNA- and PCNA-stimulated ATPase activity of RFC on the forked DNA substrate is indistinguishable from that on the base-paired primer-template. Because the model DNA substrate used in this study was very small and PCNA did not remain stably attached to it, our studies do not address whether this inhibition of the release of RFC from forked substrates is very strong and could be used as a tool to guide recombination or repair proteins rather than DNA polymerase δ to the forked DNA junction.

Acknowledgments—We thank John Majors and Tim Lohman for critical discussions during the course of this work, Sonja Gary for advice on the purification of RFC and Rfc2–5, and Rao Ayyagari for purified RPA.

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