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A Central Swivel Point in the RFC Clamp Loader Controls PCNA Opening and Loading on DNA

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Abstract

Replication Factor C (RFC) is a 5-subunit complex that loads PCNA clamps onto primer-template DNA (ptDNA) during replication. RFC subunits belong to the AAA+ superfamily, and their ATPase activity drives interactions between the clamp loader, clamp, and ptDNA, leading to topologically linked PCNA•ptDNA. We report the kinetics of transient events in *S. cerevisiae* RFC catalyzed PCNA loading, including ATP-induced RFC activation, PCNA opening, ptDNA binding, ATP hydrolysis, PCNA closing and PCNA•ptDNA release. This detailed perspective enables assessment of individual RFC-A, -B, -C, -D, -E subunit functions in the reaction mechanism. Functions have been ascribed to RFC subunits previously, based on steady state analysis of 'arginine finger' ATPase mutants; however, pre-steady state analysis provides a different view. The central subunit, RFC-C, serves as a critical swivel point in the clamp loader. ATP binding to this subunit initiates RFC activation and the clamp loader adopts a spiral conformation that stabilizes PCNA in a corresponding open spiral. The importance of RFC subunit response to ATP binding decreases as: RFC-C > RFC-D > RFC-B, with RFC-A being unnecessary. RFC-C-dependent activation of RFC also enables ptDNA binding, leading to formation of the RFC•ATP•PCNA_{open}•ptDNA complex. Subsequent ATP hydrolysis leads to complex dissociation, with RFC-D activity contributing the most to rapid ptDNA release. The pivotal role of the RFC-BCD subunit ATPase core in clamp loading is consistent with the similar central location of all three ATPase-active subunits of the *E. coli* clamp loader.

Keywords

DNA replication; RFC clamp loader; PCNA clamp; ATPase kinetic mechanism; Arginine finger

INTRODUCTION

The eukaryotic clamp loader RFC loads PCNA clamps onto DNA where they serve as processivity factors for replicative polymerases, and modulate the functions of many other proteins that work on DNA ^{1,2}. The structure and function of clamp and clamp loader proteins is highly conserved through evolution, reflecting their critical role in DNA metabolism. PCNA is a homo-trimeric ring with a central pore that accommodates duplex

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DNA^{3,4}. RFC is a hetero-pentameric complex, comprising one large (RFC-A) and four small subunits (RFC-B, -C, -D, -E) arranged in the form of a claw that binds PCNA at the base and primer-template DNA (ptDNA) within the central chamber (nomenclature note: RFC-A, -B, -C, -D, -E are RFC-1, -4, -3, -2, -5, respectively)⁵. During the loading reaction, the clamp must be opened at an inter-subunit interface to enable entry of DNA, and then closed to form a topological link between the two macromolecules (Fig. 1). The clamp loader catalyzes this reaction by bringing the clamp and DNA substrates together in an appropriate configuration for loading, and then releasing the linked clamp•DNA product. The ATPase activity of the clamp loader fuels this mechanical work.

RFC subunits belong to the AAA+ family of ATPases—proteins that utilize free energy of the ATPase reaction to manipulate other macromolecules⁶⁻⁸. The crystal structure of *S. cerevisiae* RFC complex shows that the subunits comprise three core domains (Fig. 1): the C-terminal domains pack against each other in a ring that holds the complex together at one end; the N-terminal domains form ATPase modules that are splayed in a spiral tracking the pitch of double-stranded DNA (note: the larger RFC-A subunit contains additional domains, including a C-terminal domain that packs against RFC-E)⁵. These structural features appear strongly conserved among bacterial and eukaryotic clamp loaders^{5,9-11}. Four catalytic ATPase sites are located at the RFC-E/D (D site), RFC-D/C (C site), RFC-C/B (B site) and RFC-B/A (A site) subunit interfaces. One subunit binds ATP, providing the Walker A and B motifs required for phosphate and Mg²⁺ binding, respectively, and its neighbor provides the 'arginine finger' motif comprising SRC residues. The SRC Arg is analogous to the Arg residue in GTPase-activating proteins (GAPs) that inserts into the active site of Ras and related GTP-binding proteins. It facilitates nucleotide hydrolysis by stabilizing the transition state, and is also in position to couple nucleotide binding/hydrolysis/product release into conformational changes in the protein^{12,13}. In the case of clamp loaders, it has been reported that the SRC motif is not necessary for ATP binding, but is essential for ATP hydrolysis and clamp loading¹⁴⁻¹⁷. In RFC, the RFC-E Arg contributes to the RFC-D ATP binding site at the RFC-E/D interface, and so on for the rest of the subunits, as shown in Fig. 1 (E_{SRC} in site D, D_{SRC} in site C, C_{SRC} in site B, B_{SRC} in site A); RFC-A does not have an SRC motif and the ATP binding site in RFC-E is not competent for hydrolysis^{5,18}.

This is the second of two reports tackling the question of how *S. cerevisiae* RFC uses ATP to load PCNA onto ptDNA. In the first study we focused on establishing the order and rates of transient events in the reaction, including PCNA opening and closing, ptDNA binding and release, and ATP hydrolysis and phosphate (Pi) release (see accompanying paper). Global analysis of the pre-steady state data yielded a model kinetic mechanism of this eukaryotic clamp loader, and enabled us to address specific questions about the role of individual RFC subunits in the reaction. In this study we focused on determining how ATP binding and hydrolysis by each subunit drives PCNA loading. The approach was to mutate SRC Arg residues to Ala (SAC) and assess the impact on the reaction mechanism. The results from pre-steady state analysis of mixed wild type/mutant complexes reveal the workings of each RFC subunit, and show how function and mechanistic significance depend on subunit position in the clamp loader complex.

Our report largely revises an earlier assignment of specific functions to the RFC subunits¹⁴. Significant differences between the two studies are: (a) the earlier study was based on equilibrium and steady state measurements, which provide limited information on the kinetic mechanism and, correspondingly, and the role of individual subunits therein; (b) the RFC_{SAC} complexes analyzed in the earlier study contained an N-terminal truncated version of RFC-A (ΔN_{283}), which exhibits significantly lower stoichiometric activity than the full-length wild type protein for reasons as yet unclear¹⁹.

RESULTS

Full-length wild type (SRC) and mixed wild type (SRC)/mutant (SAC) RFC complexes were purified from *E. coli* and tested for nucleotide binding activity. RFC-All_{SAC} exhibits similar ATP γ S binding affinity and stoichiometry as wild type RFC, binding up to 5 ATP γ S molecules in the presence of PCNA or both PCNA and ptDNA¹⁹ (supplementary Fig. S1). Thus, mutation of the SRC motif to SAC does not appear to disrupt overall structural integrity and nucleotide binding by RFC. The mutations are, however, likely to disrupt RFC response to ATP binding as well as ATP hydrolysis, and a previous study has shown that RFC-All_{SAC} (RFC-A Δ N₂₈₃) cannot load PCNA on ptDNA¹⁴. To understand exactly how SAC mutations disrupt the loading mechanism, we analyzed their effects on the kinetics of distinct ATP-coupled steps in the reaction.

RFC-C activity is required for PCNA opening

Stabilization of the PCNA clamp in an open state requires ATP binding to RFC, but not ATP hydrolysis. ATP binding induces conformational changes that activate RFC and are coupled to PCNA opening, prior to ptDNA binding and ATP hydrolysis (Fig. 1; see accompanying paper, Table 1). We utilized a FRET-based assay with PCNA-WC^{AEDANS} to assess the effect of SAC mutations on this early step in the reaction (PCNA-WC^{AEDANS}; W185 changed to Trp; K107 changed to Cys and labeled with AEDANS). Clamp opening results in lower FRET efficiency between the tryptophan-AEDANS donor-acceptor pair positioned across the inter-subunit interface, and clamp closure results in corresponding increase in FRET efficiency²⁰. Mixing wild type RFC and PCNA-WC^{AEDANS} with ATP results in complete PCNA opening within ~ 3 s, as shown in Fig. 2A, i (FRET efficiencies were calculated²¹ as described in the accompanying paper and normalized to the initial value of each kinetic trace to facilitate comparison between different RFC proteins). With RFC-All_{SAC}, PCNA-WC^{AEDANS} FRET remains unchanged over time, indicating that the quadruple SAC mutant cannot open PCNA (ii). The RFC-D_{SAC} (C site) mutation has the same outcome, indicating that subunit C plays a crucial role in PCNA opening (iv). Of the remaining mutants, RFC-E_{SAC} (D site) (iii) and RFC-C_{SAC} (B site) (v) exhibit fractional PCNA opening, while RFC-B_{SAC} (A site) (vi) activity is comparable to wild type RFC. These results are summarized in Fig. 5A with PCNA opening kinetic traces overlaid for all RFC complexes. The ranking of subunit importance in ATP-binding induced RFC activation and PCNA opening is as follows: C > D > B > A.

The next step was to assess the effect of RFC_{SAC} mutations on PCNA closure, which occurs toward the end of the reaction after ptDNA binding and ATP hydrolysis. Sequential mixing experiments were performed in which RFC and PCNA-WC^{AEDANS} were pre-incubated with ATP for varying times to allow clamp opening (Δ t: 0.02 – 3 s), then mixed with excess ptDNA, and the change in FRET monitored over time (FRET efficiencies were calculated²¹ as described in the accompanying paper and normalized to the 3 s value from each PCNA opening kinetic trace to facilitate comparison between the different RFC proteins). At short Δ t, initial FRET efficiency decrease (PCNA opening) and subsequent increase (PCNA closing) were detected (Fig. 2B, i), reflecting the ordered series of events—PCNA opening, ptDNA binding, then PCNA closure—in the reaction catalyzed by wild type RFC. With increasing Δ t more PCNA was opened during the pre-incubation period, thus more closure was detectable on addition of ptDNA. At Δ t: 3 s, all PCNA was opened prior to ptDNA addition and only PCNA closing was observed at a rate of ~ 7 s⁻¹. There is almost no change in PCNA-WC^{AEDANS} FRET efficiency with the RFC-All_{SAC} mutant, consistent with its inability to open PCNA (ii). Interestingly, addition of ptDNA to RFC-D_{SAC} (C site) appears to force some PCNA opening, and this fraction of open PCNA can be closed as well (iv). This result implies a less critical role for subunit C after the PCNA opening step. A similar finding holds for RFC-E_{SAC} (D site) (iii). Since the rate of PCNA closure by these two

mutants is limited by slow opening, we cannot resolve whether RFC-D_{SAC} and RFC-E_{SAC} mutations affect the PCNA closing step directly. In case of RFC-C_{SAC} (B site) (v) and RFC-B_{SAC} (A site) (vi), the fraction of PCNA opened during pre-incubation is closed on ptDNA binding, but at 2–3-fold slower rate than wild type RFC. These results are summarized in Fig. 5B with kinetic traces at Δt : 3 s overlaid for all RFC complexes. No single subunit appears to have a predominant role in PCNA closure, though their contribution can accelerate this step in the reaction.

RFC-C activity is important for ptDNA binding and RFC-D activity for ptDNA release

Since rapid ptDNA binding to RFC triggers a burst of ATP hydrolysis followed by PCNA closure, if SAC mutations affect RFC-ptDNA interaction, the rates of these subsequent steps can be affected as well. In order to investigate this possibility, we measured ptDNA binding and release as reported by fluorescence of TAMRA dye conjugated to the 3' primer end of the 40/65-nucleotide ptDNA substrate. Sequential mixing experiments were performed in which RFC and PCNA were pre-incubated with ATP for varying times to allow PCNA opening (Δt : 0.02 – 3 seconds), then mixed with ptDNA_{TAMRA}, and the change in fluorescence intensity monitored over time. As shown by the rise in ptDNA_{TAMRA} fluorescence (signal normalized to the initial value), wild type RFC binds ptDNA rapidly ($5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$; see accompanying paper, Table I), and the bound fraction increases with Δt as more RFC•ATP•PCNA_{open} complex is formed during pre-incubation (Fig. 3, i). As ATP is hydrolyzed the complex dissociates at a limiting rate of $\sim 8 \text{ s}^{-1}$, shown by the decrease in ptDNA_{TAMRA} fluorescence (note: the signal remains higher than baseline level due to excess RFC in the reaction that can bind released ptDNA_{TAMRA}; see accompanying paper, Fig. 3). RFC-All_{SAC} shows no significant binding to ptDNA (ii). Both RFC-E_{SAC} (D site) (iii) and RFC-D_{SAC} (C site) (iv) bind ptDNA at a slow rate, apparently limited by the fractional PCNA opening that occurs on addition of DNA to the reaction for these two mutants (Fig. 2B, iii, iv). The RFC-C_{SAC} (B site) (v) and RFC-B_{SAC} (A site) (vi) mutants bind ptDNA at rates comparable to wild type RFC, in line with their ability to bind and open PCNA. The relative extent of ptDNA binding (Fig. 3; maximum bound fraction compared to wild type RFC) correlates with that of PCNA opening and closing (Fig. 2; maximum open and closed fractions) for all SAC mutants, except RFC-E_{SAC} (iii), where the high ptDNA_{TAMRA} signal suggests exceptional affinity for ptDNA. These results are summarized in Fig. 5C with kinetic traces at Δt : 3 s overlaid for all RFC complexes in the presence of PCNA.

All the SAC mutants exhibit significantly lower (or negligible) complex dissociation rates compared to wild type RFC (Fig. 3), which might be due to slower PCNA closure, PCNA•ptDNA release, and/or ptDNA release steps. In order to parse these possibilities, we performed the above DNA binding experiments in the absence of PCNA. As shown in the accompanying article, ATP-induced RFC activation occurs more slowly without PCNA, thus longer pre-incubation of RFC and ATP is necessary to achieve maximal ptDNA binding ($\Delta t \geq 8 \text{ s}$). ptDNA also dissociates faster from RFC in the absence of PCNA, thus the maximum bound fraction is lower than when PCNA is present. The rate of ptDNA binding to ATP-activated wild type RFC is the same with or without PCNA ($5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). The data are shown in Fig. 5D with ptDNA binding/release kinetic traces at Δt : 8 s overlaid for all RFC complexes in the absence of PCNA. The mutants bind ptDNA at rates comparable to wild type RFC. RFC-E_{SAC} (D site), RFC-C_{SAC} (B site), and RFC-B_{SAC} (A site) also bind ptDNA to the same extent as RFC. Notably, the maximum fraction of ptDNA bound to RFC-D_{SAC} (C site) is only about half that of RFC (despite a slightly slower rate of dissociation), suggesting that RFC-C activity plays a greater role in ptDNA binding to the clamp loader than the other subunits. Another notable result is the minimal dissociation of ptDNA from RFC-E_{SAC}, which suggests that RFC-D has an important role in releasing

ptDNA following ATP hydrolysis. This finding also explains the highly stable RFC- E_{SAC} •ATP•PCNA•ptDNA complex observed in Fig. 3, iii (see also Fig. 5C). The RFC- C_{SAC} mutant (B site) also has a slightly reduced ptDNA release rate compared to wild type RFC (Fig. 5D), which, along with slower PCNA closing (Fig. 2B, v), can explain slower dissociation of the RFC- C_{SAC} •ATP•PCNA•ptDNA complex (Fig. 3, v; Fig. 5C). Finally, RFC- B_{SAC} (A site) releases ptDNA as fast as wild type RFC (Fig. 5D); therefore, the relatively slow rate of RFC- B_{SAC} •ATP•PCNA•ptDNA complex dissociation (Fig. 3, vi; Fig. 5C) is likely due to another step in the reaction such as slow PCNA closure (Fig. 2B, vi), which is proposed as a rate-limiting step in our model mechanism (see accompanying paper, Table I).

All ATPase active sites in RFC influence ptDNA-induced rapid ATP hydrolysis and Pi release

Once the RFC•ATP•PCNA_{open}•ptDNA intermediate complex is formed, ATP is hydrolyzed rapidly ($20 - 50 \text{ s}^{-1}$), followed by PCNA closure and release of products, including PCNA•ptDNA, phosphate (Pi) and ADP (see accompanying paper)¹⁹. We measured the kinetics of one of these final steps, Pi release, for all RFC mutants, in order to assess how the loss of individual subunit ATP sensing and hydrolysis activities affects the ATPase activity of RFC as a whole. Pre-steady state Pi release kinetics were measured with the fluorophore-labeled MDCC-PBP reporter protein that binds Pi rapidly and with high affinity ($k_{on} = 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$; $K_D = 0.1 \text{ }\mu\text{M}$)²². Sequential mixing experiments were performed in which RFC and PCNA were pre-incubated with ATP for varying times to allow clamp opening (Δt : 0.02 – 3 s), then mixed with excess ptDNA and MDCC-PBP, and the change in MDCC-PBP fluorescence monitored over time. The signal was converted to Pi concentration using a calibration curve and plotted *versus* time¹⁹. Fig. 4, i shows a burst of Pi release catalyzed by wild type RFC, at an apparent rate limited to $\sim 10 \text{ s}^{-1}$ and an amplitude indicating rapid ATP hydrolysis by three subunits (see accompanying paper, Table I). The steady state ATPase rate is $1.6 \text{ }\mu\text{M s}^{-1}$ ($k_{cat} = 1.1 \text{ s}^{-1}$, assuming 3 active ATPase sites per RFC at $0.5 \text{ }\mu\text{M}$ in the reaction). All the SAC mutants exhibit lower burst amplitude and/or slower rates, and RFC-All_{SAC} has no detectable activity (Fig. 4; 5E at Δt : 3s). This result means that all four nucleotide binding sites in RFC contribute to the burst, indirectly by sensing ATP binding or directly by catalyzing rapid ATP hydrolysis. RFC- D_{SAC} (iv) shows almost no burst phase, consistent with the severe defect in PCNA opening and ptDNA binding steps (preceding ATP hydrolysis) caused by disrupting the RFC-C active site (Fig. 5). RFC- E_{SAC} (iii) has lower Pi release burst amplitude and rate, and ~ 2 -fold slower steady state rate ($0.9 \text{ }\mu\text{M s}^{-1}$) than wild type RFC, probably due to defective PCNA opening and ptDNA binding (preceding ATP hydrolysis) and ptDNA release (following ATP hydrolysis) caused by disrupting the RFC-D active site (Fig. 5). RFC- B_{SAC} (vi) has almost the same Pi release profile as RFC- E_{SAC} , but in this case the underlying reasons are different. Disruption of the RFC-A active site does not affect PCNA opening or ptDNA binding (Fig. 5); thus, lower burst amplitude and rate, and slower steady-state activity of RFC- B_{SAC} ($0.95 \text{ }\mu\text{M s}^{-1}$), reflect defects in steps following ATP hydrolysis, such as PCNA closing and complex dissociation. In case of RFC- C_{SAC} (v) all steps preceding and following ATP hydrolysis are moderately affected upon disruption of the RFC-B active site, which is consistent with its intermediate Pi release kinetics profile (Fig. 5).

DISCUSSION

The eukaryotic RFC clamp loader comprises five subunits with four ATPase active sites located at the subunit interfaces. In each site, one subunit presents most of the conserved ATP binding and catalytic residues (e.g., Walker A, B motifs), while a neighboring subunit presents an arginine (SRC motif) that serves as a sensor of ATP binding and facilitates ATP

hydrolysis. Biochemical studies of bacterial and eukaryotic clamp loaders have shown that ATP (ATP γ S) binding is sufficient to form a protein-DNA complex containing an open clamp^{17,20,23-26}, and ATP hydrolysis leads to release of the clamp closed around DNA^{19,25,27-29}. In the accompanying paper we described key rate-determining events in the reaction: (a) ATP binding-induced conformational changes in RFC that enable PCNA opening and ptDNA binding, and (b) ATP hydrolysis-induced conformational changes that enable PCNA closure and PCNA•ptDNA release. Here we describe the role of each RFC subunit in these rate-determining steps, further elucidating the workings of this critical protein machine in DNA replication.

We chose to mutate the SRC motif in each subunit, expecting that this change would allow ATP binding but not hydrolysis, based on past reports^{14,15}. Moreover, the possibility of disrupting the effects of ATP binding and hydrolysis on RFC conformation provided a means to specifically investigate the ATP coupling mechanism¹³. Finally, one mystery driving our study was that the PCNA clamp is closed in the crystal structure of *S. cerevisiae* RFC-All_{SQC}•ATP γ S•PCNA complex⁵, despite evidence that ATP γ S supports PCNA opening by *S. cerevisiae* as well as *P. furiosus* RFC^{20,26}. Since the crystallized RFC had SRC motifs in all subunits mutated to SQC, we hypothesized that it was unable to respond to ATP binding and undergo the conformational changes required for PCNA opening. We changed the SRC arginines in RFC-E, -D, -C, and -B to alanine and assessed the impact by pre-steady state analysis of ATP binding-, hydrolysis- and Pi release-coupled steps in the PCNA loading reaction. Broadly, we found that RFC-C (D_{SRC}) response to ATP binding is key to assembly of the protein-DNA complex, and RFC-D (E_{SRC}) response to ATP hydrolysis is key to its disassembly; RFC-B (C_{SRC}) activity contributes modestly to complex assembly, and RFC-A (B_{SRC}) activity appears unnecessary except for slightly accelerating complex disassembly and catalytic turnover. The differing impact of SAC mutant subunits on the rate limiting steps matches their clamp loading phenotype exactly, with RFC-D_{SAC} (C site) and RFC-E_{SAC} (D site) unable to load PCNA, RFC-C_{SAC} (B site) exhibiting a moderate defect, and RFC-B_{SAC} (A site) indistinguishable from wild type RFC¹⁴. The same is true for Walker A mutants of RFC, in which the conserved lysine required for ATP hydrolysis is mutated to glutamate³⁰.

Fig. 5 provides a summary of data on intermediate steps in the reaction for wild type and mutant RFC complexes. Disruption of SRC arginine in the subunit C ATPase active site (D_{SAC}) abrogates PCNA opening completely, despite the fact that this mutation does not disrupt ATP γ S binding to RFC (supplementary Fig. S1). Indeed, RFC-All_{SAC} binds two additional ATP γ S molecules in the presence of PCNA, up to the maximum of five. This result suggests that the mutant clamp loader still binds PCNA, which in turn promotes ATP binding, but it does not undergo ATP binding-induced activation coupled with PCNA opening. Our conclusion is in accord with the RFC-All_{SQC}•ATP γ S•PCNA structure in which all nucleotide-binding sites are occupied with ATP γ S, but the clamp is not open⁵. Notably, only RFC-A, -B, and -C subunits contact the closed clamp in the structure, suggesting that RFC-All_{SQC} cannot progress from this initial interaction to one in which RFC-D and -E also contact the clamp, as required for its opening³¹; a similar proposal was made based on structural analysis of an *Archaeoglobus fulgidus* RFC sub-complex³². The impact of disrupting the ATPase sites varies as RFC-C > RFC-D > RFC-B > RFC-A, from no opening to full opening activity (Fig. 5A). We propose that ATP binding at the RFC-D/C interface initiates conformational changes that propagate through RFC-D and -E and create a compatible binding surface for the open clamp³³. Thus, the RFC-All_{SQC}•ATP γ S•PCNA structure is an early intermediate in the RFC activation pathway, wherein initial (partial) interaction between RFC and closed PCNA promotes additional ATP binding and ‘twisting’ of RFC until the proteins are mutually trapped in a right-handed spiral. This conclusion is in accord with the marked acceleration of ATP-induced RFC activation in the presence of

PCNA (see accompanying paper)¹⁹. A previous study had concluded that all RFC_{SAC} mutants, including RFC-All_{SAC} (RFC-A Δ N₂₈₃) can open PCNA, based on fluorescent labeling of Cys81 at the PCNA inter-subunit interface that is exposed on clamp opening¹⁴. We suggest that this assay could not resolve the substantive differences in PCNA opening kinetics because of prolonged incubation with excess fluorophore (30 seconds) during which even a transiently open clamp could be labeled. The same study also showed that the ATPase activity of triple RFC_{SAC} (RFC-A Δ N₂₈₃) mutants containing active RFC-C or RFC-D subunits was stimulated by PCNA. This result complements our finding that RFC-C and RFC-D response to ATP binding is critical for PCNA opening.

Only the RFC-D_{SAC} (C site) mutation reduces ptDNA binding to RFC (Fig. 5D). This finding is consistent with equilibrium binding measurements that could not detect interaction between ptDNA and RFC-D_{SAC} (RFC-A Δ N₂₈₃), while the other mutants had similar or slightly lower affinity than wild type RFC¹⁴. We suggest that the ATP-induced conformational changes initiating at subunit C optimize RFC contacts with ptDNA, and that no other subunit has a predominant role in creating/maintaining the helical dsDNA binding track along the interior of RFC. In the presence of PCNA, the effects of SAC mutations on ptDNA binding kinetics appear more significant (Fig. 5C); however, apparent slow ptDNA binding to RFC-E_{SAC} (D site) and RFC-C_{SAC} (B site) could be simply due to slow PCNA opening and/or slow RFC activation that occur prior to ptDNA binding and, conversely, rapid ptDNA binding to RFC-B_{SAC} (A site) is consistent with its wild type-like PCNA opening activity. Only in the case of RFC-D_{SAC} (C site), the apparent slow rate can be attributed definitively to defective PCNA opening/RFC activation (Fig. 5A) as well as defective ptDNA binding (Fig. 5D).

The kinetic model developed in the accompanying article shows that rapid ATP hydrolysis after ptDNA binding is followed by a relatively slow step at or prior to PCNA closure, PCNA•ptDNA and Pi dissociation (Fig. 1). We have proposed that this step involves relaxation of RFC back to an inactive conformation that has low affinity for open PCNA and ptDNA. Pi release kinetics indicate that all four active sites contribute to the ATPase activity, directly by catalysis or by allosteric effects on other sites, since the pre-steady state burst amplitude and rates are lower for all mutants compared to wild type RFC (Fig. 5E). Note that for the mutants, burst amplitude is not a direct measure of the number of ATP molecules hydrolyzed, since these complexes exhibit sub-stoichiometric activity prior to the ATP hydrolysis step. The steady state ATPase rates of wild type and mutant complexes appear quite similar, but that is not surprising since they all bind ptDNA, which alone can stimulate the rate to $\sim 2 \mu\text{M s}^{-1}$ ¹⁹. In case of RFC-D_{SAC} (C site) the apparent rates of PCNA closure, PCNA•ptDNA and Pi release are slow, but we cannot resolve if RFC-C contributes specifically to these late steps in the reaction, because of its essential role in the early steps of RFC activation, PCNA opening (Fig. 5A) and ptDNA binding (Fig. 5D). The RFC-D subunit plays a significant role in the early steps as well, but we have direct evidence that disruption of ATP hydrolysis at this site (E_{SAC}) also slows down ptDNA release (Fig. 5D). The PCNA closure, PCNA•ptDNA and Pi release kinetics of RFC-C_{SAC} confirm a modest role for RFC-B in the reaction, starting with the PCNA opening step. Unlike the other subunits, ATP binding to RFC-A has no detectable impact on the early steps, but the RFC-B_{SAC} mutation does lower the rates of PCNA closure, PCNA•ptDNA and Pi release slightly, suggesting that the site contributes to RFC catalytic turnover. Analysis of *A. fulgidus* RFC also indicates that the ATPase activity of the large subunit (equivalent to RFC-A) is involved in catalytic turnover^{17,32}.

Analysis of the pre-steady state burst of ATP hydrolysis and Pi release by wild type RFC reveals that at least 3 ATPase sites hydrolyze ATP rapidly, and at the same apparent rate, once the RFC•ATP•PCNA_{open}•ptDNA complex is formed (see accompanying paper)¹⁹. It is

possible that the fourth site hydrolyzes ATP at a slower rate that is difficult to distinguish from steady state, or that some aspect of our assay conditions masks the activity of this site. The mutant data indicate that ATP binding and hydrolysis by the RFC-BCD core is necessary and sufficient to complete the PCNA loading reaction; therefore, we propose that ptDNA triggers ATP hydrolysis at these three active sites to initiate complex dissociation. The RFC-A subunit makes extensive contacts with PCNA⁵; thus, the ATPase activity of this site (perhaps at a slower rate) could further assist in PCNA closure, PCNA•ptDNA release and catalytic turnover. This interpretation is consistent with an earlier study of RFC Walker A site mutants, which also showed that only the RFC-BCD core ATPase activity is required for PCNA loading³⁰. A comparison of the effects of SRC mutations in *E. coli* γ complex^{16,34} reveals significant similarities with the RFC clamp loader mechanism described here. In this case, the 3 central γ subunits possess ATPase active sites while δ' and δ do not (δ' , γ_1 , γ_2 , γ_3 , δ are positional equivalents of RFC-E, -D, -C, -B, -A)¹⁰. Both δ'_{SAC} (γ_1 site) and γ_{SAC} (γ_2 and γ_3 sites) mutants suffer significant loss in β clamp binding affinity and clamp opening, consistent with the significant loss in PCNA clamp opening by RFC- E_{SAC} (D site) and RFC- D_{SAC} (C site) mutants. The γ_{SAC} mutants (γ_2 and γ_3 sites) also lose affinity for DNA, and although the contribution(s) of γ_2 and/or γ_3 to DNA binding cannot be parsed, this result is also consistent with our finding that RFC- D_{SAC} (C site) has lower ptDNA binding activity than wild type RFC. Importantly, the γ complex SRC mutant study revealed that the 3 ATPase active sites located in the clamp loader core are necessary and sufficient for loading β onto ptDNA, which mirrors our finding about the BCD subunit core in RFC¹⁶.

In conclusion, we have found that RFC subunit function varies with position in the clamp loader complex (Fig. 5F). RFC-C serves as a critical swivel point in the center of RFC, and its response to ATP binding propagates conformational change towards the peripheral subunits. As a result, RFC adopts an active spiral form creating an extensive binding interface at which all subunits, including RFC-D and -E, contact an open PCNA clamp. RFC-C dependent RFC activation also facilitates interaction with ptDNA. Subsequent rapid ATP hydrolysis by at least three subunits, likely the RFC-BCD core, prompts relaxation of RFC back into a form with low affinity for ptDNA and PCNA. At this stage the conformational change appears to propagate in reverse, i.e., from the peripheral RFC-D and -E subunits. RFC-A involvement in these ATP-coupled conformational dynamics appears less significant, suggesting that the work done by the D/E arm of the RFC spiral is more important than the A/B arm while loading PCNA on DNA.

MATERIALS AND METHODS

RFC and PCNA, PCNA-WC^{AEDANS}, and MDCC-PBP proteins were prepared as described in the accompanying paper. For RFC complexes with mutant SRC motifs, two pLANT2/RIL-RFC-A/E plasmids containing full-length RFC-A were constructed by replacing a *Dra*I fragment of pLANT2/RIL-RFC-^{HK} Δ N-A/E and pLANT2/RIL-RFC-^{HK} Δ N-A/ E_{SAC} ¹⁴ with a *Dra*I fragment coding for the N-terminal 282 amino acids of RFC-A subunit from pLANT2/RIL-RFC-A/^{HK}E³⁵. Co-expression with pET11a-RFC-B $_{SAC}$ /C/D, pET11a-RFC-B/C $_{SAC}$ /D or pET11a-RFC-B/C/D $_{SAC}$ plasmids yielded RFC complexes with individual SAC mutant subunits (B: Arg157; C: Arg160; D: Arg183; E: Arg184). ptDNA substrates were prepared as described in the accompanying paper.

Kinetic measurements of PCNA opening/closing, ptDNA binding/release, and phosphate release by the mixed wild-type/mutant RFC $_{SAC}$ complexes were performed and the data analyzed as described in the accompanying paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PCNA proliferating cell nuclear antigen

RFC	replication factor C
ptDNA	primer-template DNA
Pi	inorganic phosphate
TAMRA	5-(and 6-) carboxytetramethylrhodamine
AEDANS	5-[2(acetyl)aminoethyl-]aminonaphthalene-1-sulfonate
MDCC-PBP	7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl coumarin-labeled phosphate binding protein
FRET	Förster resonance energy transfer

Fig. 1.

RFC structure and kinetic mechanism. (A) A computationally derived model of RFC bound to ATP γ S and open PCNA³³. (B) Schematic of the ATPase sites at RFC subunit interfaces. Each subunit, except RFC-E, contains an active site to which a neighboring subunit contributes an SRC motif (depicted as R). (C) Close-up view of the D site showing the RFC-D P-loop and the RFC-E SRC motif. (D) Schematic of key steps in the clamp loading mechanism reported in the accompanying paper. (I) Binding of 2-3 ATP molecules to RFC is followed by (II) RFC interaction with PCNA and additional binding of 1-2 ATP molecules¹⁹, and slow activation steps involving conformational changes in RFC and PCNA that lead to (III) a stable open clamp complex. (IV) ptDNA binds rapidly and with high affinity to the activated RFC•ATP•PCNA complexes, (V) triggering ATP hydrolysis. (VI) Next, another slow step involving conformational changes in RFC and PCNA leads to closure of the clamp around ptDNA, (VII) release of products (Pi , PCNA•ptDNA) and catalytic turnover.

Fig. 2.

The RFC-C site is critical for PCNA opening and loading on DNA. Opening/closing of PCNA clamp is measured by change in FRET between tryptophan (donor; $\lambda_{\text{EX}} = 290$ nm) and AEDANS (acceptor; $\lambda_{\text{EM}} > 450$ nm) at the PCNA-WC^{AEDANS} inter-subunit interface. (A) Mixing of RFC and PCNA with ATP results in a decrease in FRET efficiency over time as PCNA opens (panels i – vi: wild type and RFC_{SAC} mutant data). RFC-D_{SAC} (C site) is almost incapable of opening PCNA like RFC-All_{SAC}, and RFC-B_{SAC} (A site) shows almost no defect. (B) Pre-incubation of RFC, PCNA with ATP (Δt : 0.02 – 3 s) followed by ptDNA addition results in FRET increase as PCNA closes around DNA. At shorter Δt (e.g., 0.02 s; grey trace) only a fraction of PCNA is open, and both clamp opening (FRET decrease) and

closing (FRET increase) are detected. At longer Δt (e.g., 3 s; dark blue trace), all PCNA is open, and only clamp closing is detected for wild type, RFC-C_{SAC}, and RFC-B_{SAC}. ptDNA binding to RFC-D_{SAC} and RFC-E_{SAC} can induce some PCNA opening. RFC-B_{SAC} and RFC-C_{SAC}, which open PCNA to a significant extent, exhibit ~2-fold slower PCNA closing. Final reactant concentrations: 0.6 μM RFC, 0.25 μM PCNA, 0.25 μM ptDNA (when present) and 0.5 mM ATP. PCNA opening/closing rates for wild type RFC are in the accompanying paper (Table I).

Fig. 3.

RFC_{SAC} mutants exhibit defects in RFC•ATP•PCNA•ptDNA association and dissociation. ptDNA binding to RFC in the presence of PCNA is measured by change in fluorescence intensity of 3' primer TAMRA-labeled ptDNA (λ_{EX} 550 nm, λ_{EM} > 570 nm). Pre-incubation of RFC, PCNA with ATP (Δt : 0.02 – 3 s) followed by ptDNA_{TAMRA} addition results in signal increase on ptDNA binding, and decrease on dissociation (panels i – vi: wild type and RFC_{SAC} mutant data). The bound ptDNA fraction increases with Δt . The mutants apparently bind ptDNA at slower rates than wild type, except RFC-B_{SAC}, and release ptDNA slowly as well. Notably, RFC-E_{SAC} appears to bind and retain ptDNA at a high level, despite poor PCNA opening activity. Final reactant concentrations: 0.1 μM RFC,

0.4 μM PCNA, 0.04 μM ptDNA_{TAMRA} and 0.5 mM ATP. ptDNA binding/release rates for wild type RFC are in the accompanying paper (Table I).

Fig. 4. Disruption of the RFC-C ATPase active site abrogates rapid ATP hydrolysis and Pi release. RFC-catalyzed Pi release is measured by change in fluorescence intensity of MDCC-PBP reporter. Pre-incubation of RFC, PCNA with ATP (Δt : 0.02 – 3 s) followed by ptDNA addition results in a rapid burst of Pi release and a linear steady state phase (panels i – vi: wild type RFC and RFC_{SAC} mutant data). The burst amplitude increases with Δt , reflecting slow formation of the RFC•ATP•PCNA_{open} intermediate complex preceding ptDNA binding, ATP hydrolysis and Pi release. RFC_{SAC} mutants exhibit lower burst amplitude (reduced stoichiometry of ATP hydrolysis) than wild type RFC, especially RFC-D_{SAC} (C site) where the burst is barely detectable. The steady state ATPase rates vary by about 2-

fold. Final reactant concentrations: 0.5 μ M RFC, 1 μ M PCNA, 2.5 μ M ptDNA, 0.5 mM ATP and 10 μ M MDCC-PBP. Pi release rates for wild type RFC are in the accompanying paper (Table I).

Fig. 5. Overlay of kinetic data for wild type and mutant RFC, and a model for RFC subunit contribution to PCNA loading. Kinetic traces are shown for (A) PCNA opening (Δt : 3 s), (B) PCNA closing (Δt : 3 s), (C) DNA binding/release in the presence (Δt : 3 s) and (D) in the absence (Δt : 8 s) of PCNA, and (E) Pi release (Δt : 3 s), for wild type and mutant proteins. The data are color coded by the affected active site: RFC-E_{SAC} (D site): green, RFC-D_{SAC} (C site): red, RFC-C_{SAC} (B site): purple, RFC-B_{SAC} (A site): pink, RFC-All_{SAC}: grey, RFC: blue. These data highlight the predominant early effect of the RFC-D_{SAC} mutation on PCNA opening and pDNA binding, and the late effect of the RFC-E_{SAC} mutation on pDNA release. (F) Model of RFC subunit contributions to PCNA loading on DNA. Both RFC complex and PCNA ring are depicted in linear form, the ATPase active sites are depicted as circles, and the significance of each site at different steps in the reactions is indicated by color: black > grey > white. The color scheme does not imply any particular sequence of ATP hydrolysis.