

# A Heterotrimeric PCNA in the Hyperthermophilic Archaeon *Sulfolobus solfataricus*

## Short Article

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### Summary

The sliding clamp, PCNA, of the archaeon *Sulfolobus solfataricus* P2 is a heterotrimer of three distinct subunits (PCNA1, 2, and 3) that assembles in a defined manner. The PCNA heterotrimer, but not individual subunits, stimulates the activities of the DNA polymerase, DNA ligase I, and the flap endonuclease (FEN1) of *S. solfataricus*. Distinct PCNA subunits contact DNA polymerase, DNA ligase, or FEN1, imposing a defined architecture at the lagging strand fork and suggesting the existence of a preformed scanning complex at the fork. This provides a mechanism to tightly couple DNA synthesis and Okazaki fragment maturation. Additionally, unique subunit-specific interactions between components of the clamp loader, RFC, suggest a model for clamp loading of PCNA.

All forms of cellular life possess sliding clamps to enhance the processivity of their replicative DNA polymerases (Ellison and Stillman, 2001). In bacteria this activity is brought about by the actions of the  $\beta$  clamp, whereas in eucarya and archaea the analogous functions are provided by the proliferating cell nuclear antigen, PCNA. In addition to enhancing processivity of the polymerase, PCNA has been observed to act as a scaffold for the assembly of accessory processing factors such as DNA ligase I and the flap endonuclease, FEN1 (reviewed in Warbrick, 2000). Therefore, it appears that PCNA acts as a central organizer for the assembly of proteins during lagging strand synthesis. Intriguingly, these additional proteins bind to the same site on PCNA, the interdomain connector loop, as does the DNA polymerase. Current models invoke a sequential recruitment of these factors to PCNA during lagging strand synthesis (Warbrick, 2000; MacNeill, 2001; Bae et al., 2001). Although there is only very limited sequence homology between the  $\beta$  clamp and PCNA and despite the fact that the  $\beta$  clamp

is a homodimer and PCNA is a homotrimer, the overall morphology of these complexes is remarkably similar. Both the  $\beta$  clamp and PCNA form toroidal rings with a central hole, through which DNA is proposed to pass (O'Donnell et al., 2001). The ring-like structures of the sliding clamps must therefore be opened and closed to allow entry of DNA to this central cavity. This reaction is performed by the clamp loader; the  $\gamma$  complex in bacteria, and replication factor C (RFC) in archaea and eucarya (Ellison and Stillman, 2001). The mechanisms underpinning the loading of the bacterial clamp have been extensively studied at both the biochemical and structural level. The recent determination of 3D crystal structures of the  $\gamma$  complex bound to the  $\beta$  clamp has added weight to a model in which the  $\gamma$  complex facilitates clamp loading by opening one of the interfaces between the two  $\beta$  subunits (reviewed in Ellison and Stillman, 2001). This reaction is stimulated by the binding of ATP and the subsequent hydrolysis of ATP facilitates resealing of the ring around DNA.

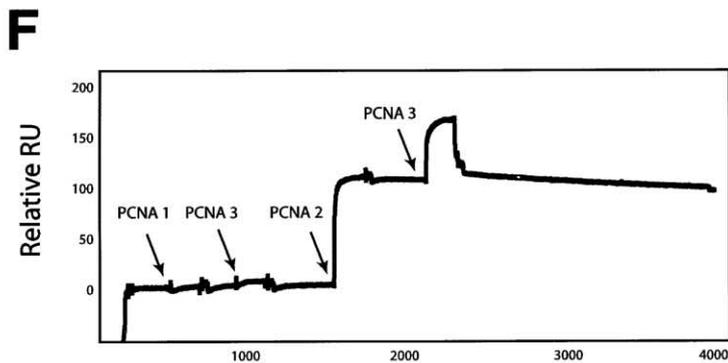
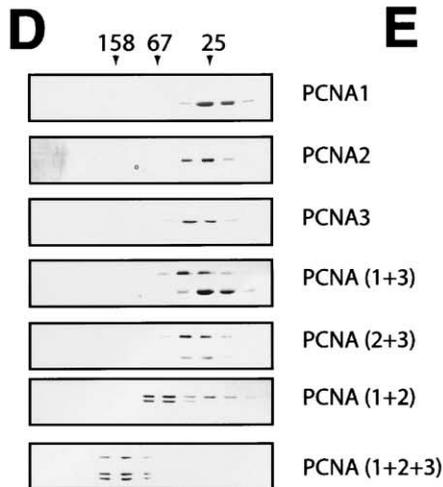
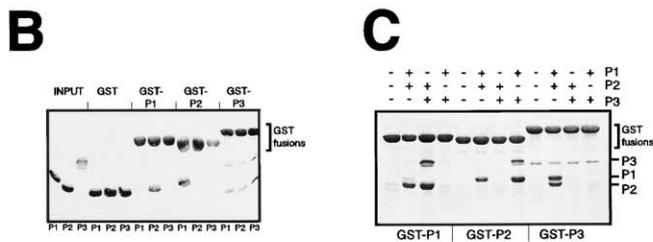
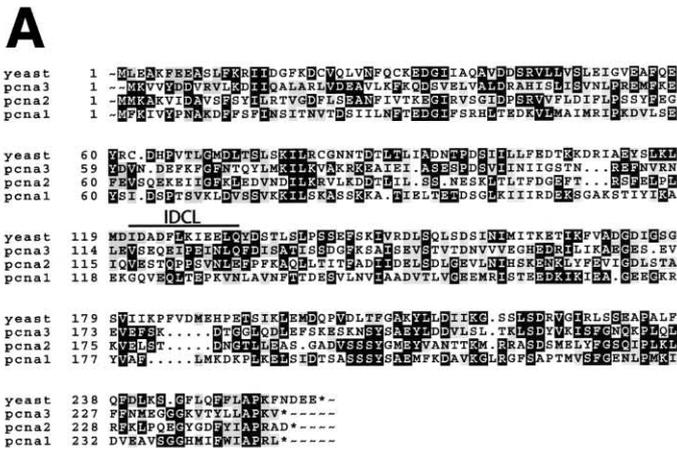
While a great deal of mechanistic detail is known about the bacterial clamp loading process, less is known about the mechanisms of clamp loading of archaeal and eucaryal PCNA. This has been particularly hard to analyze as the homotrimeric nature of PCNA presents a considerable technical challenge to assessing individual interactions between PCNA subunit(s) and RFC components.

### Results and Discussion

We have initiated a systematic study of the DNA replication apparatus of the hyperthermophilic archaeon *Sulfolobus solfataricus*. As with other archaea, the *Sulfolobus* machinery resembles the core components of the eucaryal DNA replication apparatus (Bernander, 2000; Cann and Ishino, 1999). As part of this project we have identified genes for three PCNA homologs in the genome sequence of this hyperthermophilic archaeon (Figure 1A; She et al., 2001). We have termed these PCNA1, 2, and 3, respectively. We sought to determine whether these three PCNA homologs form distinct homotrimers or a heterotrimer. PCNA1, 2, and 3 were expressed and purified individually, either as fusion proteins with Glutathione S transferase (GST) or a hexa histidine (His) tag. Next GST PCNA1, 2, or 3 were tested for their ability to interact with purified His-tagged PCNA1, 2, or 3. Using pairwise combinations of GST and His tagged PCNAs we could detect strong interactions between PCNA1 and PCNA2 (Figure 1B). PCNA3 was unable to interact with any of PCNA1, 2, or 3 individually. This indicates that none of the PCNAs homo-multimerize to any significant degree; however, PCNA1 and PCNA2 can interact with each other. Next we tested multiple combinations of PCNA subunits in the pull down assay (Figure 1C). We now found that PCNA3 could be pulled down in the reactions that contained GST-PCNA1 and PCNA2 and GST-PCNA2 and PCNA1. Additionally, GST-PCNA3 was able to pull down the PCNA1-PCNA2 dimer. Thus, the

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PCNA of *S. solfataricus* is a heterotrimer of PCNA1, 2, and 3. Furthermore, these data indicate that there is a defined order of assembly with the formation of a

PCNA1-PCNA2 heterodimer being a prerequisite for recruitment of PCNA3. The heterotrimeric nature of the *S. solfataricus* PCNA was supported by gel filtration

Figure 1. *Sulfolobus solfataricus* PCNA Is a Heterotrimer

(A) Alignment of the predicted protein sequence of *S. solfataricus* PCNA homologs with budding yeast PCNA. PCNA1, 2, and 3 are encoded by genes annotated as SSO0397, SSO1047, and SSO0405 on the *Sulfolobus solfataricus* genome web site (<http://www-archbac.u-psud.fr/projects/sulfolobus/>).

(B) Interactions between PCNA1 and PCNA2. The indicated GST fusion proteins were bound to glutathione sepharose and incubated with 20  $\mu$ g of purified recombinant PCNA1, 2, or 3. Following extensive washing, beads were boiled in SDS-PAGE loading buffer and proteins detected by electrophoresis followed by staining with Coomassie brilliant blue dye.

(C) Combinations of PCNA1, 2, and 3 were incubated with the GST-PCNA beads and treated as above.

(D) Ten micrograms of the indicated PCNA subunits were chromatographed on a Superose 12 column, and fractions were analyzed by SDS-PAGE and stained with Coomassie blue. The positions of peak elution of marker proteins are indicated.

(E) Immunoprecipitation with antisera specific for either PCNA1, 2, or 3 from 100  $\mu$ g of *S. solfataricus* whole cell extract. Immunoprecipitates were assayed by Western blotting with the PCNA1, 2, or 3 antisera.

(F) Sensorgram of sequential injections of PCNA subunits on a sensor chip coupled to immobilized GST-PCNA1. Injection points are indicated.

analysis on a Superose 12 column. The individual PCNA subunits elute from the column as monomer sized, as do mixes of PCNA1 and 3 and PCNA 2 and 3. However, PCNA1 and 2 form a dimer sized complex and the combination of PCNA1, 2, and 3 yields an elution profile in agreement with formation of a heterotrimer in which all three subunits are present in a 1:1:1 stoichiometric ratio (Figure 1D). Additionally, immunoprecipitation assays were performed on a whole cell extract prepared from *S. solfataricus* cells using anti-sera specific for individual PCNAs (Figure 1E). These assays revealed coimmunoprecipitation of all three PCNA subunits, indicating the presence of heterotrimeric PCNA in vivo. Surface plasmon resonance experiments were performed to further analyze the formation of the PCNA heterotrimer. PCNA1 was immobilized on the surface of a sensor chip and one of PCNA1, PCNA2, or PCNA3 flowed over the cell. We were unable to detect significant PCNA1-PCNA1 or PCNA1-PCNA3 interactions but were able to detect strong binding of PCNA1 and PCNA2 (Figure 1F). Quantitation of this interaction revealed a KD for this interaction of  $1.2 \times 10^{-11}$  M (data not shown). This interaction has an extremely slow off-rate ( $k_d = 1.7 \times 10^{-5} \text{ s}^{-1}$ ). Once formed the PCNA1-2 dimer was then able to recruit PCNA3 (Figure 1F). It was therefore possible to introduce PCNA3 to the PCNA1-2 dimer on the chip and calculate a KD of  $2.7 \times 10^{-7}$  M for the binding of the third component of the ring. This figure is approximately 4 orders of magnitude lower than that of the PCNA1-2 interaction. This dramatic difference was largely as a result of a rapid off rate for PCNA3 ( $k_d = 5.3 \times 10^{-2} \text{ s}^{-1}$ ). The order of assembly that we detect and the ready reversibility of PCNA3 binding may have significant implications for the clamp loading mechanism (see below).

The heterotrimeric nature of the *S. solfataricus* PCNA led us to speculate that distinct PCNA subunits may contact distinct replication-associated factors. Previous work has established that eucaryal PCNA can interact with DNA polymerase, DNA ligase I, and FEN1 during lagging strand synthesis, leading to a model of sequential recruitment of these factors to PCNA during lagging strand synthesis (e.g., see Bae et al., 2001; MacNeill, 2001; Tom et al., 2001). We examined the interactions of *S. solfataricus* homologs of these factors with individual PCNA subunits. We found that the major B-type polymerase (polB1) of *S. solfataricus* interacted most strongly with PCNA2 and also more weakly with PCNA3 (Figure 2B, upper panel). Previous studies of eucaryal and bacterial sliding clamps have defined a consensus sequence for clamp interaction sites (Dalrymple et al., 2001). We find a strongly conserved match to this sequence in the extreme N terminus of polB1s from *Sulfolobus* species (Figure 2A). Deletion of this motif resulted in a significant reduction in the interaction with PCNA2 and PCNA3 (Figure 2B, lower panel). Additionally, we tested the ability of individual PCNA subunits or the assembled heterotrimer to stimulate the DNA synthetic capability of the DNA polymerase. We find that the heterotrimeric PCNA, but not individual subunits, is able to significantly stimulate polymerase activity; furthermore this stimulation is dependent on the N-terminal motif in polB1 (Figure 2C). Our finding is in marked contrast to a report by Pisani and colleagues that indicated that high concentrations of individual PCNA1 and PCNA3

could stimulate the polymerase (De Felice et al., 1999). Because we have demonstrated that these individual subunits do not form trimers and because we cannot detect any interaction between PCNA1 and polB1, we assume the weak stimulation previously reported was a nonspecific effect.

Next, we tested the ability of the PCNA subunits to interact with *S. solfataricus* DNA ligase I. We identified a candidate interaction motif in the N-terminal region of this protein (Figure 2D). We detected a unique interaction between PCNA3 and DNA ligase I (Figure 2E, upper panel). Furthermore, a deletion construct, lacking the first 30 amino acids of the ligase, did not interact detectably with PCNA3 (Figure 2E, lower panel). As with DNA polB1, we found that the PCNA heterotrimer could stimulate ligase activity, but that individual subunits could not (Figure 2F, left panel). This stimulation was dependent on the presence of the N-terminal interaction motif (Figure 2F, right panel).

Next, we tested the interaction of FEN1 with the PCNA subunits. Using the yeast 2-hybrid assay and GST pull downs, we could detect a strong interaction between PCNA1 and FEN1. Additionally, a very weak interaction between FEN1 and PCNA3 could be detected (Figures 2G and 2H). Both these interactions were dependent on a candidate PCNA interaction motif in the extreme C terminus of FEN1 (Figures 2G, 2H, and 2I). Finally, we tested the effect of addition of PCNA subunits or assembled heterotrimer on the flap cleavage activity of FEN1. We could not detect any significant cleavage by FEN1 alone or in the presence of individual PCNA subunits. However, when PCNA heterotrimer was added to the reaction we could detect a significant level of product (Figure 2J). Thus, PCNA1, 2, and 3 display unique or highly preferential interactions with FEN1, DNA polymerase, and DNA ligase1, respectively. Next, we tested whether FEN1, ligase, and polymerase could interact with the PCNA ring as well as with individual subunits. To test this hypothesis we performed pull down assays using glutathione sepharose coupled to GST, GST-PCNA1, (GST-PCNA1)-PCNA2 complex and (GST-PCNA1)-PCNA2-PCNA3 complex (Figure 2K). The heterodimeric and heterotrimeric complexes were assembled with hexahistidine tagged PCNA2 and PCNA3 on GST-PCNA1 as described in Figures 1B and 1C. These matrices were then incubated with purified recombinant hexahistidine tagged polB1, FEN1, and DNA ligase1. In agreement with our previous data we found that FEN1, but not polB1 or ligase, interacted with GST-PCNA1. Significantly, however, both FEN1 and polB1 were bound by the (GST-PCNA1)-PCNA2 beads and all three proteins, polB1, ligase, and FEN1, were retained by beads containing (GST-PCNA1)-PCNA2-PCNA3 (Figure 2K). These data suggest that all three proteins might be able to simultaneously associate with the PCNA ring. To test this hypothesis, we made GST-FEN1 and tested for its ability to interact with polB1 and DNA ligase. As can be seen in Figure 2L, we can only detect interactions with ligase and polymerase in the presence of the PCNA ring, suggesting that PCNA is acting as a bridge between these factors. In light of these data, we propose that in *Sulfolobus*, DNA polB1, ligase I, and FEN1 are constitutively associated with the PCNA heterotrimer allowing a preassembled processing complex to scan along DNA

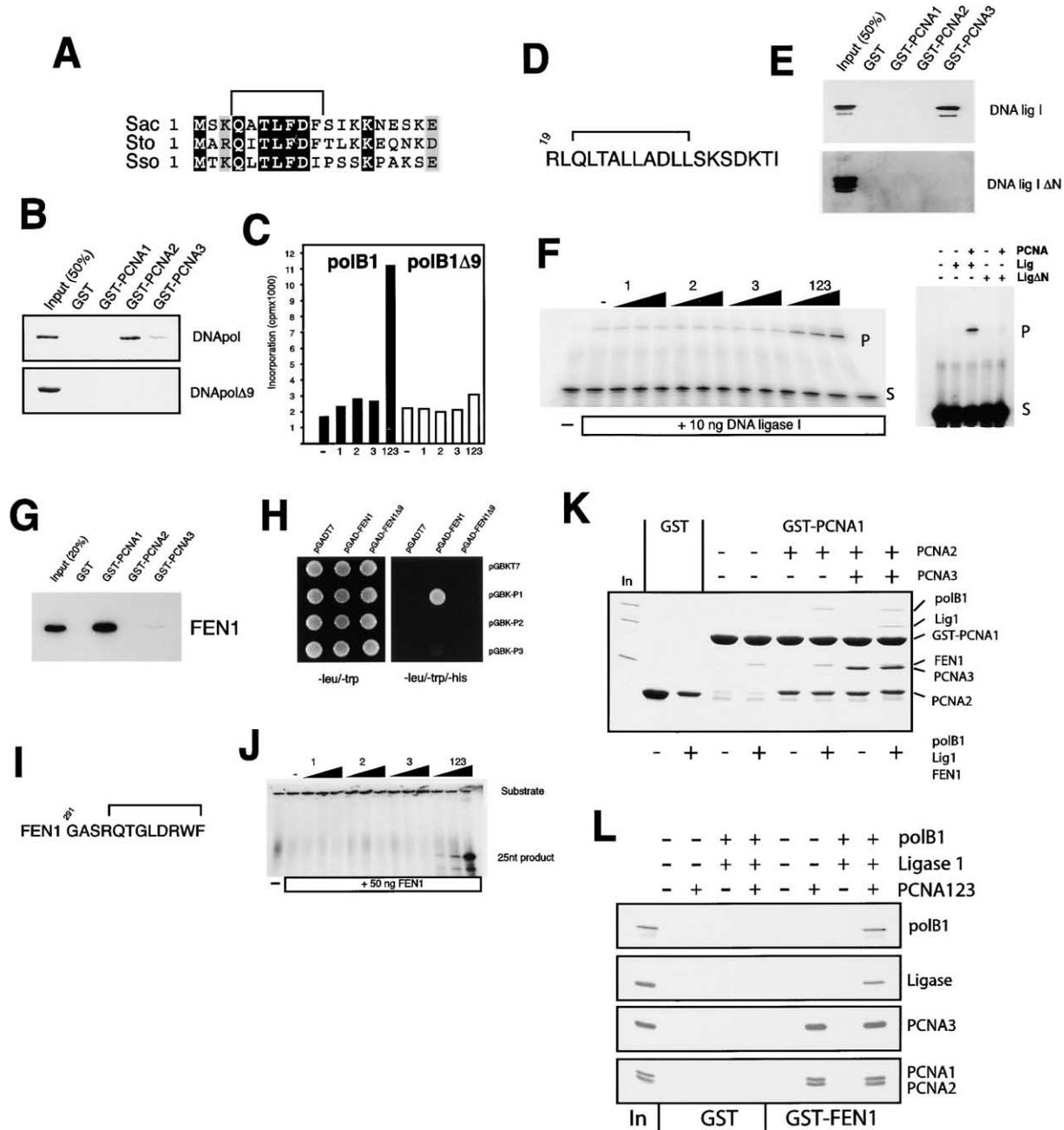


Figure 2. Interactions between PCNA1, 2, or 3 with Replication-Associated Proteins

(A) Sequence alignment of the N-terminal region of DNA polB1 from *S. solfataricus*, *S. acidocaldarius*, and *S. tokodaii*. The bracketed region indicates a candidate PCNA interaction motif.

(B) Interactions between purified DNA polB1 and PCNAs were detected using GST pull-down approaches. Ten micrograms of the indicated GST fusion protein was immobilized on GST sepharose and incubated with 10  $\mu$ g DNA polB1 (upper panel), or DNA polB1 lacking the first 9 amino acid residues. Following extensive washing, beads were boiled and proteins detected by SDS-PAGE and staining with Coomassie.

(C) PCNA heterotrimer stimulates dNTP incorporation by DNA polB1. Linearized primed M13 template was incubated with 5 ng of PCNA1, 2, 3 or heterotrimer in the presence of 1 ng DNA polB1 or polB1Δ9 with 200  $\mu$ M dCTP, dGTP, dTTP and 20  $\mu$ M dATP (containing 10  $\mu$ Ci  $\alpha^{32}$ P-dATP) for 5 min at 60°C min. Incorporation was measured by filter binding assay.

(D) Sequence of candidate PCNA interaction motif in DNA ligase 1.

(E) Interaction of DNA ligase I with PCNA3. GST pull-downs were performed as described in (B), with the exception that 5  $\mu$ g of wild-type (upper panel) or N-terminally truncated (lower panel) ligase I was present in the reaction and bound protein detected by Western blotting with an anti-hexa his tag antibody (Novagen).

(F) Ligase assays were performed as described (Srisakanda et al., 2000) with, in the left-hand panel, 10 ng of DNA ligase and either 50, 25, or 12.5 ng of PCNA1, PCNA2, PCNA3, or PCNA123 heterotrimer as indicated. The position of substrate (S) and product (P) are indicated. In the experiment in the right-hand panel, samples contained 10 ng of either wild-type or N-terminally truncated ligase in the presence or absence of 100 ng PCNA heterotrimer.

(G) FEN1 interacts preferentially with PCNA1. GST pull-downs were performed with 5  $\mu$ g purified recombinant FEN1. FEN1 was detected using the anti-His tag antibody.

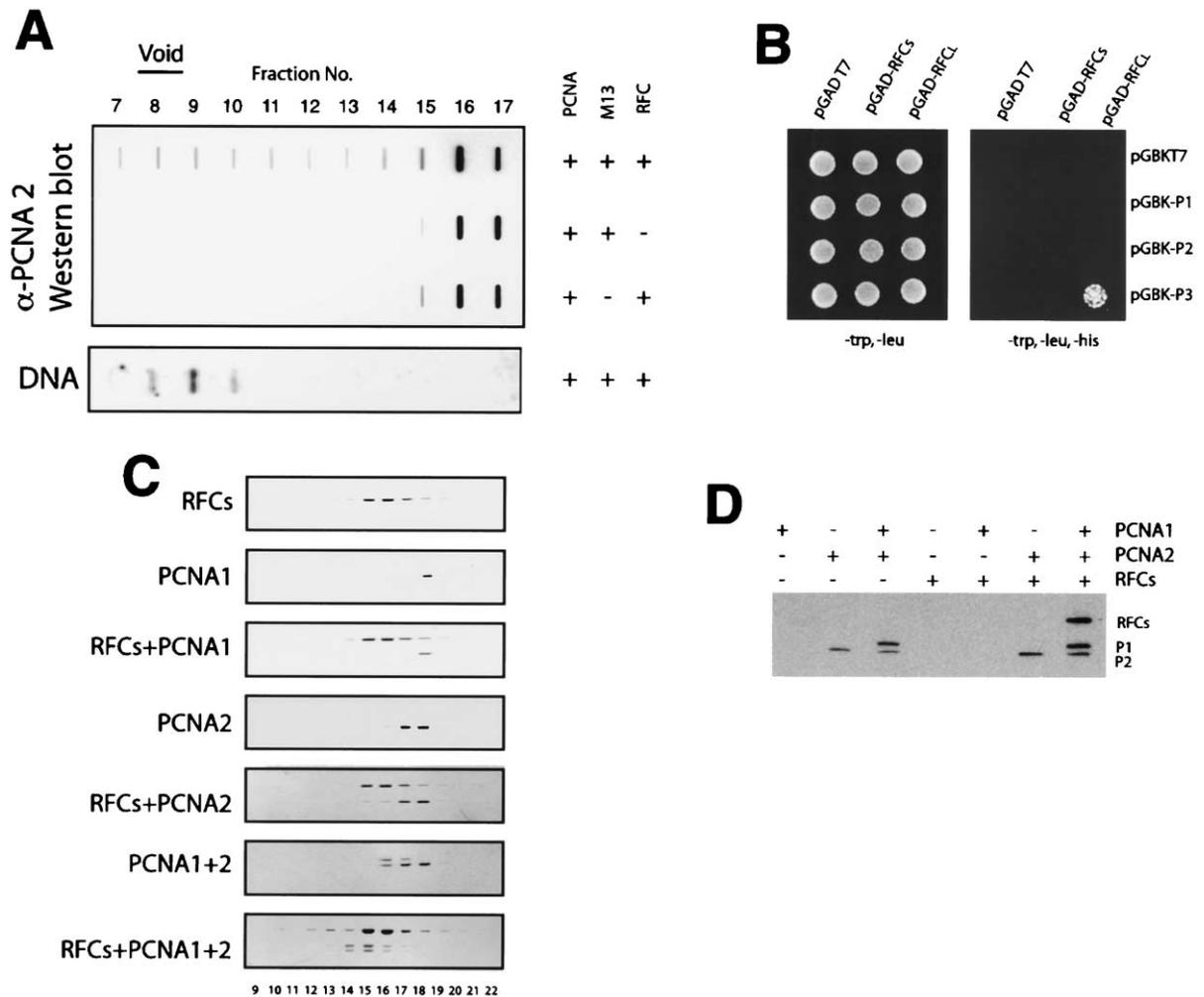


Figure 3. Differential Interactions between RFC Components and PCNA Subunits

(A) Loading of PCNA on primed M13 by RFC. Loading assays were performed as described in the Experimental Procedures and fractionated on a Superose 12 column. Fifty percent of the volume of the indicated fractions was blotted onto nitrocellulose membrane using a slot-blot manifold and PCNA detected by Western blotting with anti-PCNA 2 antisera. DNA was detected by slot-blotting followed by Southern hybridization with a radiolabeled probe generated by random priming of M13 DNA.

(B) Two-hybrid analysis of RFC large subunit, PCNA subunit interactions. Yeast were transformed with the indicated plasmids, grown, and spotted as described in Experimental Procedures.

(C) Gel filtration analysis on a Superose 12 column of 10  $\mu$ g PCNA1, 10  $\mu$ g PCNA2, and 40  $\mu$ g RFC small subunit and combinations thereof. The positions of peak elution of size markers are indicated at the top and fraction numbers are shown below. Proteins were detected by staining with Coomassie brilliant blue.

(D) Coimmunoprecipitation of RFCs, PCNA1, and PCNA2. Ten micrograms of the indicated proteins was mixed and then incubated with 0.5  $\mu$ l of rabbit polyclonal antisera raised against PCNA2 for 1 hr at 4°C. Immune complexes were recovered on Protein A Dynabeads and proteins present were identified by Western blotting with the anti-His tag antibody.

(H) Yeast two-hybrid analysis of FEN1-PCNA interactions. In this assay, interacting proteins confer the ability to grow on media lacking histidine and adenine. Yeast were transformed with the indicated plasmids, grown, and spotted as described.

(I) Sequence of the candidate PCNA interaction motif in FEN1.

(J) Heterotrimeric PCNA stimulates FEN1 activity. FEN1 was incubated with a synthetic flap substrate as described (Tom et al., 2000) in the presence of PCNA1, 2, or 3 or PCNA123 heterotrimer. The release of the 25 nucleotide flap was assayed by electrophoresis on a 12% polyacrylamide gel containing 8 M urea.

(K) Interactions between polB1, ligase 1, FEN1, and PCNA complexes. Five micrograms of GST or GST-PCNA1 was immobilized on cgluathione sepharose and incubated with 5  $\mu$ g of PCNA2 and/or 3 as indicated. Following washing, the beads were incubated with a mix of 3  $\mu$ g each of polB1, FEN1, and Ligase1. Following washing, proteins bound were identified by SDS-PAGE and staining with Coomassie brilliant blue.

(L) PCNA bridges between FEN1 and ligase and polymerase. Five micrograms GST or GST-FEN1 on beads was incubated with 2  $\mu$ g of PCNA heterotrimer and/ or 1  $\mu$ g of ligase and polB1 as indicated. Following washing, bound proteins were identified by electrophoresis and Western blotting with specific anti-sera as indicated.

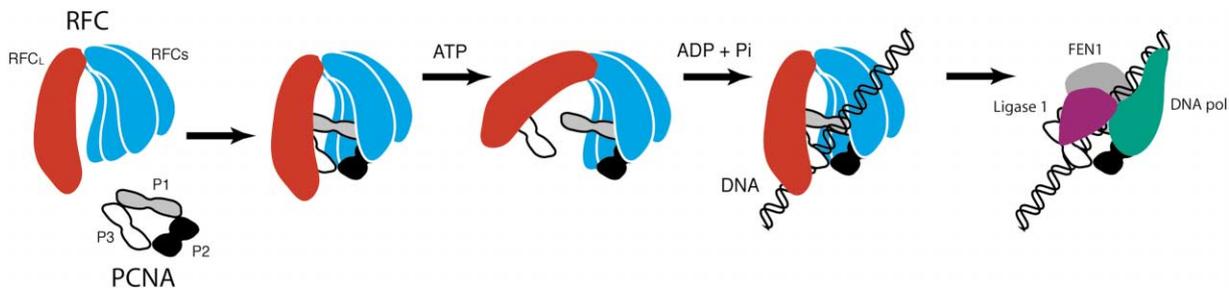


Figure 4. Model for the Mechanism of Clamp Loading

RFC complex binds to the PCNA heterotrimer, the RFC large subunit contacts PCNA3, and the RFC small subunit tetramer binds to PCNA1 and 2. Upon ATP binding a conformational shift is observed in RFC that leads to opening of the PCNA ring, by separating PCNA3 from the PCNA1-2 dimer. Upon ATP hydrolysis the ring is resealed around DNA. PCNA then acts as a scaffold for assembly of FEN1, DNA ligase, and DNA polB1.

with the DNA polymerase. This would facilitate a tight coupling of DNA synthesis and Okazaki fragment processing. In mechanistic terms this may be akin to the association of RNA processing factors with elongating eucaryal RNA polymerase II (Proudfoot et al., 2002).

We next wished to test whether the innate asymmetry of the heterotrimeric PCNA might extend to its interactions with the clamp loader, RFC. In archaea, RFC comprises a single large subunit (RFC<sub>L</sub>) in complex with a homotetramer of a small subunit (RFC<sub>S</sub>) (Cann and Ishino, 1999; Kelman and Hurwitz, 2000). We attempted to purify individual small subunit and large subunit and the pentameric complex. Although we could readily obtain large quantities of small subunit tetramer and the large subunit-small subunit complex, we were unable to isolate stable large subunit on its own. To determine whether the RFC complex could act as a clamp loader for PCNA, we modified a previously described clamp loading assay (Kelman and Hurwitz, 2000). In this assay, we incubated RFC with PCNA, ATP, and partially duplex M13 DNA, then subjected the reaction mix to gel filtration chromatography. The elution profile of PCNA was followed using anti-PCNA2 antisera in Western blotting. Comparison of the profile of the reaction with all components with reactions lacking either M13 or RFC indicates retention of PCNA in void volume fractions only in reactions that possess RFC, ATP, DNA, and PCNA complex (Figure 3A). Thus, *Sulolobus* RFC acts as a clamp loader for PCNA.

To address the potential interactions between RFC and PCNA, we employed the yeast two hybrid assay. In this approach, we could detect an interaction between PCNA3, the last subunit to assemble into the PCNA trimer, and RFC<sub>L</sub>. No interaction between PCNA3 and RFC<sub>S</sub> was detected, nor could we detect any interactions between either PCNA1 or PCNA2 and either component of RFC (Figure 3B).

Although no interactions between either PCNA1 or PCNA2 and RFC were detected in the two hybrid assay, we further investigated potential interactions using biochemical approaches. Accordingly, we incubated PCNA1 or PCNA2 with RFC<sub>S</sub> and subjected the reaction to fractionation on a Superose 12 gel filtration column. The elution profiles of PCNA1 and PCNA2 were essentially identical regardless of the presence of RFC<sub>S</sub>, indicating that there are no stable interactions between these com-

ponents. Next we mixed PCNA1 and 2 prior to incubation with or without RFC<sub>S</sub>. Strikingly, we now found that the PCNA1-2 dimer formed a stable complex with the RFC<sub>S</sub> tetramer of considerably higher apparent molecular mass than that of the PCNA1-2 dimer alone. These data indicate that the tetrameric RFC<sub>S</sub> complex interacts specifically with a PCNA1-2 dimer and not with either subunit alone (Figure 3B). We further tested this hypothesis by performing immunoprecipitation experiments on mixes of PCNA1, PCNA2, and RFC<sub>S</sub>. Various combinations of these proteins were mixed and then immunoprecipitation performed with antisera raised against PCNA2. The presence of proteins in the immunoprecipitate was assayed using a commercially available anti-hexahistidine monoclonal antibody that recognizes the His-tag on all three proteins. Consistent with the gel filtration data, we could only detect immunoprecipitation of RFC<sub>S</sub> when both PCNA1 and PCNA2 were present (Figure 3C).

These interaction data, taken together with the above data on the in vitro order of assembly of PCNA, and in conjunction with previous structural studies on ATP-induced movements in RFC (Shiomi et al., 2000), suggest a model for the loading of the PCNA clamp by RFC (Figure 4). In the model, RFC binds to PCNA, making contacts between RFC<sub>L</sub> and PCNA3 and additionally between the PCNA1-2 dimer and the RFC<sub>S</sub> tetramer. The binding of ATP by RFC induces a conformational alteration in RFC, altering the spacing between large and small subunits, thereby levering PCNA3 subunit off the PCNA ring. In this regard, it is particularly interesting to note that we find that PCNA3 is the final component to join the heterotrimeric *Sulfolobus* PCNA ring in the in vitro assembly reaction. Finally, DNA is bound and ATP is hydrolysed, thereby completing the cycle and resealing the PCNA ring shut. The PCNA ring then serves as scaffold for the simultaneous assembly of DNA polymerase, ligase, and flap endonuclease at the lagging strand fork. Although the heterotrimeric structure of *Sulfolobus* PCNA that has facilitated this analysis appears to be unique, we anticipate that our findings will apply to other systems. Specifically, we anticipate that analogous interactions to those we have described will play pivotal roles in lagging strand processing and RFC-mediated clamp loading in eukaryotes. We note that some other archaea possess several genes for PCNA subunits; how-

ever, whether these form heterotrimers is unknown (Daimon et al., 2002). In addition there is a specialized sliding clamp in eucarya involved in DNA repair; the 911 complex, composed of the homologs of *Schizosaccharomyces pombe* Rad9, Rad1, and Hus1 (Thelen et al., 1999). Although the precise functions of this complex are not yet understood, in light of our data it is tempting to speculate that each PCNA-like protein may be capable of specific and unique interactions with other components of the DNA-damage signaling machinery. It is generally accepted that the hyperthermophilic archaea are representative of the most ancient archaea and therefore closest to the progenitor of modern eucarya and achaea. It is possible therefore that the heterotrimeric *Sulfolobus* PCNA may represent an evolutionary fossil, indicative of the progenitor sliding clamp that over evolutionary time has diverged and specialized to become the homotrimeric replication associated PCNA and the heterotrimeric 911 complex found in present day eucarya. In addition to shedding light on the evolution of this class of replication and repair accessory factors, it is anticipated that the *Sulfolobus* PCNA will provide a highly useful tool for dissection of the functional and structural interactions underpinning clamp loading.

#### Experimental Procedures

##### Plasmid Constructs

Restriction sites were introduced flanking open reading frames during pcr amplification from *Sulfolobus solfataricus* genomic DNA. Oligonucleotide sequences are available on request. For protein expression the following plasmids were generated. (1) His tag fusion protein plasmids: PCNA1 cloned into NcoI, XhoI sites of pET33b (Novagen); PCNA2 cloned into NdeI, XhoI sites of pET30a (Novagen); PCNA3 cloned into NdeI, XhoI sites of pET30a; DNAP and DNAP $\Delta$ 9 cloned into NcoI, XhoI sites of pET33b; Ligase I and Ligase I  $\Delta$ N cloned into NdeI, XhoI sites of pET30a; FEN1 and FEN1 $\Delta$ C cloned into NdeI, XhoI sites of pET33b; RFC small subunit NcoI Sall digest cloned into NcoI XhoI sites of pET33b; (2) GST fusion protein plasmids: The PCNA subunits and FEN1 were cloned into the BamHI, XhoI sites of pGex4T3 (Amersham biosciences); (3) Plasmids for two-hybrid analysis: DNA binding domain fusions; PCNA1 cloned into NcoI/Sall sites of pGBKT7 (Clontech); PCNA2 cloned into NdeI/Sall sites of pGBKT7; PCNA3 cloned into NdeI/Sall sites of pGBKT7; Activation domain plasmids: RFC small subunit amplified adding BamHI and Sall sites and cloned into BamHI, XhoI sites of pGADT7 (Clontech); RFC large subunit cloned into NdeI, XhoI sites of pGADT7; FEN1 and FEN1D9 cloned into NdeI, XhoI sites of pGADT7. The identity of inserts in all plasmids was verified by DNA sequencing.

##### Two-Hybrid Analysis

Yeast AH109 were transformed with the plasmids indicated in the figure legends. Liquid cultures were grown overnight in medium lacking leucine and tryptophan. Expression of fusion proteins was verified by Western blotting according to the manufacturer's instructions (Clontech Matchmaker manual). For plating, cells were diluted to  $OD_{600} = 0.1$  and 5 ml of the dilution was spotted on a -TRP -LEU plate and on a -TRP -LEU -HIS plate. Cells were grown for 2-3 days at 30°C.

##### Protein Purification

Expression plasmids were transformed into *E. coli* strain Rosetta (DE3) pLysS (Novagen). His tag proteins were purified by chromatography over Ni-NTA Agarose (Qiagen) followed by dialysis into 10 mM Tris (pH 8.0), 150 mM NaCl, and chromatography over a HiTrap heparin column. GST fusion proteins were immobilized on Glutathione sepharose according to the manufacturer's instructions (Amersham Bioscience). The PCNA heterotrimer was purified by mixing

equimolar amounts of PCNA1, 2, and 3 and then subjecting the mix to gel filtration on a Superose 12 column in 10 mM Tris (pH 8.0), 150 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.1% Triton X-100.

##### GST Pull-Down Assays

Five to ten micrograms of the indicated GST fusion protein immobilized on glutathione sepharose (bed volume approximately 10  $\mu$ l) was incubated with the indicated amount (typically 1-5  $\mu$ g) of candidate interaction partner in 400  $\mu$ l of TBSTM (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20, and 5 mM MgCl<sub>2</sub>). Incubations were shaken at room temperature for 30 min and beads recovered by centrifugation. Beads were then washed five times with 1 ml of TBSTM. Proteins retained on beads were eluted by boiling in SDS-PAGE loading buffer and resolved on 11.25% SDS-PAGE gels. Proteins were detected by either staining with Coomassie brilliant blue or Western analysis as indicated in the figure legends.

##### FEN1 Assays

These assays were performed as described (Tom et al., 2000) using substrate D4:U3:T1. Assays used the conditions described with the exception that they were performed at 50°C.

##### RFC Loading Assay

Partially duplex M13mp18 DNA was prepared by primer extension with T7 DNA polymerase in the presence of limiting concentrations of dNTPs. Loading reactions were performed in 50  $\mu$ l containing 20 mM Tris (pH 7.5), 5 mM DTT, 80 mM MgCl<sub>2</sub>, 4% glycerol, and 2 mM ATP, and 200 ng PCNA heterotrimer. Where indicated, reactions contained 500 ng RFC and/or 200 ng M13. Reactions were incubated at 70°C for 10 min followed by gel filtration on a 3.2  $\times$  30 Superose 12 column. Eighty microliter fractions were collected and 40  $\mu$ l of each fraction immobilized on nitrocellulose membrane using a slot blot vacuum manifold. The presence of PCNA was determined by Western blotting.

##### Biacore Analyses

Anti-GST antibody was immobilized on a CM5 sensor chip using amine coupling according to Biacore protocols. 250 RU of purified GST-PCNA were immobilized and varying concentrations of PCNA1, 2, or 3 injected. In the experiment shown in Figure 1F, 280 nM PCNAs were flowed over the sensor chip at a rate of 10  $\mu$ l/min.

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