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Replication Factor C from the Hyperthermophilic Archaeon *Pyrococcus abyssi* Does Not Need ATP Hydrolysis for Clamp-loading and Contains a Functionally Conserved RFC PCNA-binding Domain

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The molecular organization of the replication complex in archaea is similar to that in eukaryotes. Only two proteins homologous to subunits of eukaryotic replication factor C (RFC) have been detected in *Pyrococcus abyssi* (*Pab*). The genes encoding these two proteins are arranged in tandem. We cloned these two genes and co-expressed the corresponding recombinant proteins in *Escherichia coli*. Two inteins present in the gene encoding the small subunit (*Pab* RFC-small) were removed during cloning. The recombinant protein complex was purified by anion-exchange and hydroxyapatite chromatography. Also, the *Pab* RFC-small subunit could be purified, while the large subunit (*Pab* RFC-large) alone was completely insoluble. The highly purified *Pab* RFC complex possessed an ATPase activity, which was not enhanced by DNA. The *Pab* proliferating cell nuclear antigen (PCNA) activated the *Pab* RFC complex in a DNA-dependent manner, but the *Pab* RFC-small ATPase activity was neither DNA-dependent nor PCNA-dependent. The *Pab* RFC complex was able to stimulate *Pab* PCNA-dependent DNA synthesis by the *Pab*-family D heterodimeric DNA polymerase. Finally, (i) the *Pab* RFC-large fraction cross-reacted with anti-human-RFC PCNA-binding domain antibody, corroborating the conservation of the protein sequence, (ii) the human PCNA stimulated the *Pab* RFC complex ATPase activity in a DNA-dependent way and (iii) the *Pab* RFC complex could load human PCNA onto primed single-stranded circular DNA, suggesting that the PCNA-binding domain of RFC has been functionally conserved during evolution. In addition, ATP hydrolysis was not required either for DNA polymerase stimulation or PCNA-loading *in vitro*.

Keywords: replication factor C; archaea; hyperthermophile; *Pyrococcus abyssi*; PCNA-binding domain

Abbreviations used: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); hPCNA, human proliferating cell nuclear antigen; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LB, Luria–Bertani broth; MCM, minichromosome maintenance protein; M13ds, double-stranded M13mp18 DNA; M13ssc, single-stranded circular M13mp18 DNA; *Pab*, *Pyrococcus abyssi*; *Pab* DNA pol I, *Pyrococcus abyssi* family B DNA polymerase; *Pab* DNA pol II, *Pyrococcus abyssi* family D DNA polymerase; *Pab* PCNA, *Pyrococcus abyssi* proliferating cell nuclear antigen; *Pab* RFC complex, *Pyrococcus abyssi* replication factor C; *Pab* RFC-small, *Pyrococcus abyssi* replication factor C small subunit; *Pab* RFC-large, *Pyrococcus abyssi* replication factor C large subunit; ph-PCNA, phosphorylable derivative of the human proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride membrane; RPA, replication protein A; SSB, single-stranded DNA-binding protein.

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Introduction

Chromosome DNA replication occurs in a strikingly similar manner in diverse organisms, requiring for a DNA replication complex, a DNA polymerase holoenzyme that is functionally conserved between eukarya, bacteria and the bacteriophage T4.[1] The holoenzyme is composed of three essential protein components: (i) a DNA polymerase; (ii) a processivity factor (the sliding-clamp); and (iii) a multi-protein complex (the clamp-loader). DNA polymerases alone lack high processivity, but upon association with their respective sliding-clamps, they can duplicate a long chromosome continuously and accurately. The only known physiological mechanism by which the sliding-clamp can be assembled onto DNA is through its ATP-dependent interaction with the clamp-loader. The clamp-loading process has been studied in all systems.[2–10]

In eukaryotes, the replication factor C (RFC) clamp-loader is formed by five subunits.[11] The RFC clamp-loader loads the homotrimeric proliferating cell nuclear antigen (PCNA) clamp onto DNA in an ATP-dependent manner.[12] While PCNA associates with DNA polymerase δ,[13] or ε,[14] processive DNA synthesis occurs[15] and RFC is thought to dissociate from DNA.[16] In all systems tested, the kinetic mechanisms of holoenzyme assembly require many intermediate structural complexes and ATP hydrolysis.[3,4,17] The exact nature of the ATP hydrolysis events and the conformational changes required for loading the ring-shaped sliding-clamp onto DNA in these organisms have recently been described in detail.[6–9]

The cellular structure and metabolism of archaea, the third domain of life,[18] are similar to those of bacteria. However, archaeal DNA replication systems are homologous to those of eukaryotes. The complete archaeal genome sequencing[19–24] has allowed identification of putative homologues of eukaryotic DNA replication proteins. Interestingly, DNA replication mechanisms seem to be quite different within the archaeal subdomains. Whereas the crenarchaeotes, a subdomain of archaea, contain two[25] or three[23] PCNA homologues, and two or three family B DNA polymerases,[26,27] the euryarchaeotes possess only one PCNA homologue,[28,29] one family B and one family D DNA polymerase, respectively.[30–32] The evolution of PCNA homologues has been characterized by one or two gene duplication events, which are believed to have occurred after the split of the crenarchaeal and euryarchaeal lineages.[33] In archaea, two genes with homology to RFC have been identified. They share sequence homology with their eukaryotic counterparts. Seven amino acid motifs (referred to as boxes II–VIII) in both RFC subunits are highly conserved. However, so far, experimental evidence that shows the involvement of the archaeal proteins in DNA replication or that they play other cellular roles, such as in DNA repair, is lacking.

Recent structural and biochemical studies of archaeal clamp-loaders have revealed surprising discrepancies. While the *Sulfolobus solfataricus* and *Pyrococcus furiosus* clamp-loaders form apparently a heteropentamer,[34,35] the RFC complex from the euryarchaeote *Methanobacterium thermoautotrophicum* appears to be a hexamer.[36]

P. abyssi (*Pab*) is an anaerobic hyperthermophilic euryarchaeon with an optimal growth temperature at 96 °C.[37] The entire genome sequence of *P. abyssi* was published recently†. Amino acid sequence comparisons with eukaryotic homologous proteins showed that DNA replication components (DNA polymerases, PCNA, RFC and RPA) are conserved. Three open reading frames (ORFs) encoding putative homologues of eukaryotic RFC subunits and PCNA have been identified. Here, we describe the isolation and characterization, of an RFC complex homologue from *P. abyssi* (*Pab* RFC). This complex is a fully active clamp-loader, able to load PCNA onto a DNA heteroduplex and to stimulate the PCNA-dependent primer-extension abilities of *Pab* DNA polymerase (pol) II. Our data suggest that the PCNA-binding domain of the RFC is functionally conserved from archaea to human.

Results

Identification of the clamp-loader complex and genetic organization of *P. abyssi*

A BLASTX analysis of the genomic database of *P. abyssi* identified two putative genes homologous to the large and small eukaryotic RFC subunits: *Pab* RFC-small and *Pab* RFC-large. These two genes are arranged in tandem on the *P. abyssi* chromosome. They form a 5765 bp operon (Figure 1) and are separated by a short (11 bp) intergenic sequence. Analysis of the location of the *Pab* RFC cluster genes showed that they are located within an archaeal origin of replication spot[38] containing genes homologous to the eukaryotic counterparts involved in the initiation (such as Cdc6/Orc1) and elongation of DNA replication (the two subunits of the new archaeal-type family D DNA polymerase[32]). In addition, this region is comprised of genes encoding for a putative DNA helicase and for a Rad51 homologue (Figure 1, top).

Pab RFC-small and *Pab* RFC-large are 4314 bp and 1440 bp long, respectively. The *Pab* RFC-small gene is only 993 bp long after the excision of the two inteins. Putative transcriptional and translational signals have been identified (Figure 1, bottom).

The amino acid residue sequences of the *Pab* RFC subunits were compared together with those of archaeal, eukaryotic and prokaryotic homologues (Figure 2). Similarities were found in seven regions

† http://www.genoscope.cns.fr/Pab/

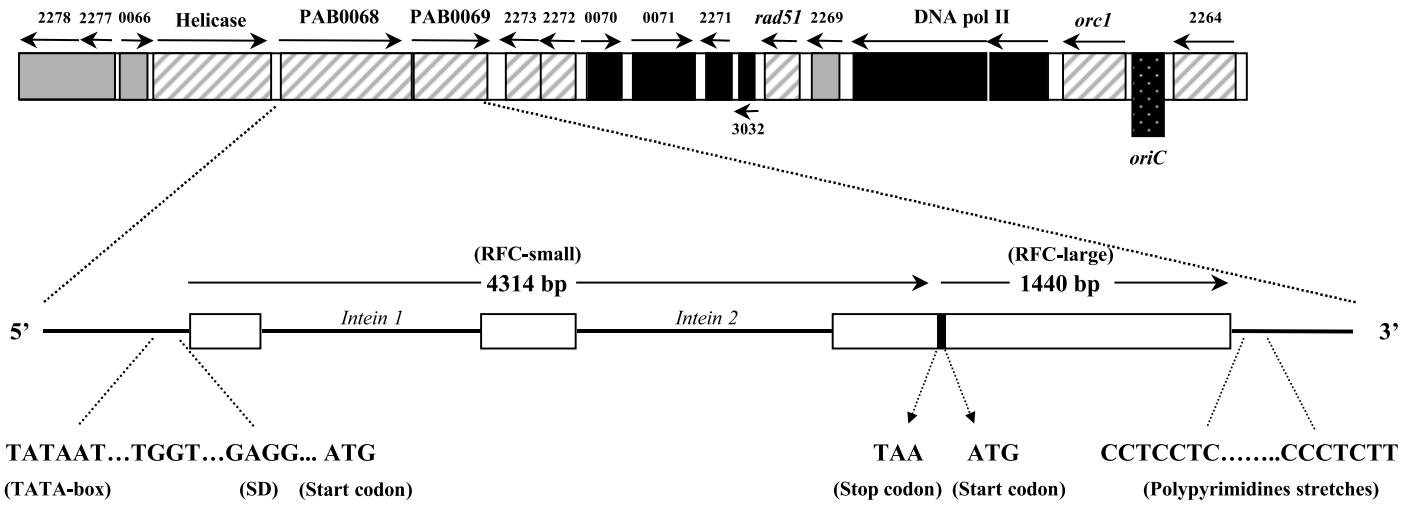
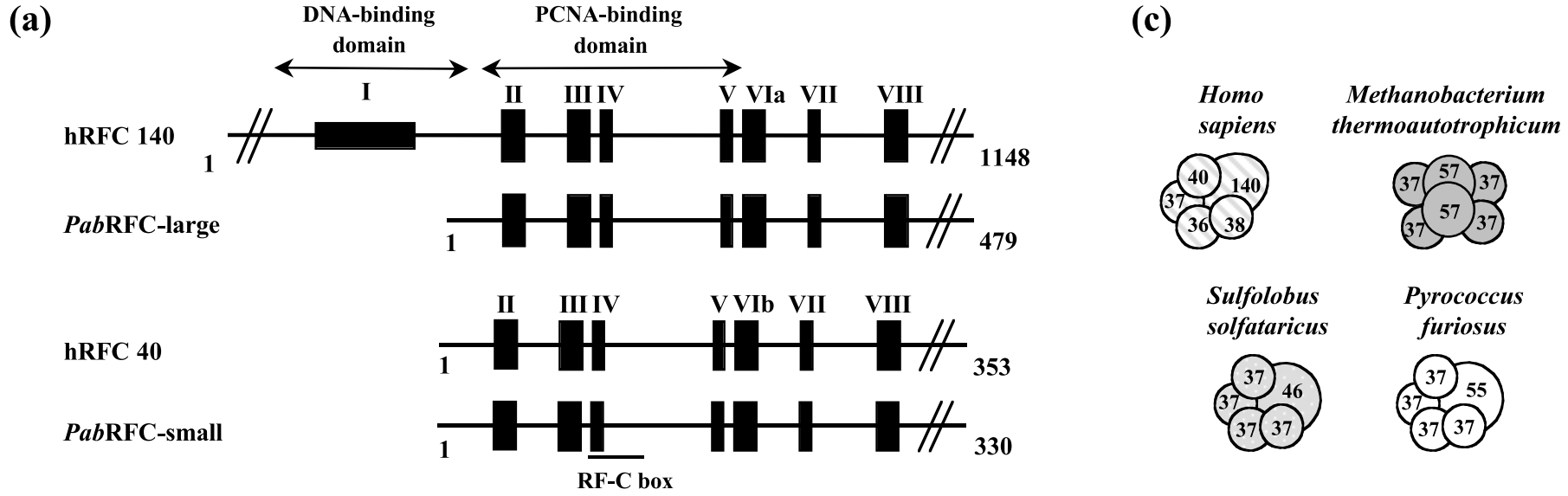


Figure 1. Physical map of the *P. abyssi* origin of replication locus containing the RFC genes PAB0068 and PAB0069. The putative genes encoding the helicase and Rad51 protein are indicated. The family D DNA polymerase, encoded by two subunits, and a putative initiation factor (Orc1) were identified upstream of the *P. abyssi* candidate origin of replication.[38] *Pab* RFC-small and *Pab* RFC-large are arranged in tandem, in an operon (5765 bp). The putative transcription and translation signals are shown at the bottom: from left to right, a putative eukaryotic-like TATA-box, a putative prokaryotic-like Shine–Dalgarno motif (SD), the *Pab* RFC-small gene start and stop codons, the *Pab* RFC-large gene start codon, and the putative eukaryotic-like transcription termination sequence (polypyrimidine stretches). Archaeal-specific genes are shown in black, bacterial-specific genes in gray, and eukaryotic-like genes are hatched.[38] 0066: alanyl-tRNA synthetase gene; 2264: methylthioadenosine phosphorylase gene; 2273: molybdenum cofactor biosynthesis protein gene; 2277: IMP dehydrogenase gene; 2278: lacZ expression regulatory protein gene. Genes 0070, 0071, 2269, 2271, 2272 and 3032 code for hypothetical proteins.



(b)

Boxes	II	III	IV	V	VIb	VII	VIII
Motifs		*P-Loop*		*DEAD-box*			
hRF-C 140	LWVDKYKPTSL	KAALLSGPPGVGKTTT	V-ELNASD	H-ALIMDEVD	GMAGNEDRGGIQEL	HYC	QDIRQVLHNL
*Pab*RF-C2	PWVEKYRPRRL	KALLLAGPPGSGKTTT	VIELNASD	K-IIFLDEAD	NIEP---SGAPEIK	DRA	QDLRAAINDL
*Sso*RF-C L	QWFLKYRPRSL	KAVLLHGPPGVGKTVL	L-EMNASD	LKLILLDEVD	GINVREDTGAIQGI	NKA	GDARYAINIL
*Mth*RF-C L	SWTEKYRPGSF	KPLLLVGPPGTGKTTL	L-ELNASD	LKLIILDEVD	GIHGNEDRGGVQAI	PRC	GDLRSAINDL

	VIa				
hRF-C 40	PWVEKYRPVKL	NIIIAGPPGTGKTTS-	LELNASN	-KIIILDEAD	MTDGAGGALRRTME-	SRC	QGDMRQALNNLQ
*Pab*RF-C1	PWVEKYRPQKL	HLLFAGPPGVGKTTA-	LELNASD	-KIIFLDEAD	LTQDAQQALRRTME-	SRC	EGDMRRAINILQ
*Sso*RF-C S	LWAEKYRPKTL	PHLIFAGPPGTGKTTA	LELNASD	FKVVLLDEAD	MTADAQQALRRTMEL	SRT	-GDMRKSINIL-
*Mth*RF-C S	PWVEKYRPQKL	PHLLFTGPAGVGKTTA	LELNASD	FRIIFLFEVD	MTKDAQHALRREME-	SRC	-GDLRKAINLL-

Figure 2. Comparison of archaeal and eukaryotic RFC subunits. (a) Conserved RFC boxes among *Pab* RFC and human RFC. The corresponding regions are numbered on top. The length (in amino acid residues) is shown on the right. The RFC box that includes box IV is indicated at the bottom. The arrows map the DNA-binding domain (369–480) and the PCNA-binding domain (481–728) in the hRFC 140. (b) Amino acid sequence similarities within the RFC boxes. *Mth* RFC (from *M. thermoautotrophicum*), *Sso* RFC (from *S. solfataricus*) and hRFC (from human) were compared to the *Pab* RFC. The P-loop and DEAD-box are indicated. (c) Structure of the active forms of RFC complexes from human,[40] *M. thermoautotrophicum*,[36] *S. solfataricus*[34] and *P. furiosus*.[35]

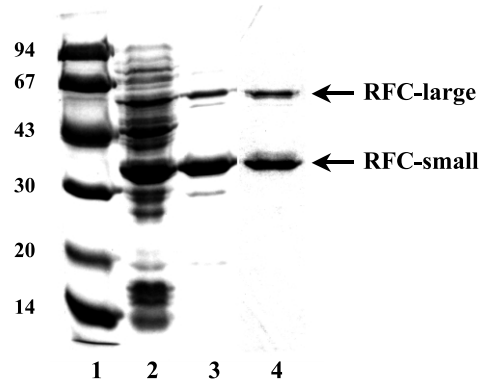


Figure 3. Co-expression of the *Pab* RFC subunits in *E. coli* produced a stable soluble complex. Purification of *Pab* RFC subunits was performed as outlined in Materials and Methods. Soluble proteins from each step of purification were separated by SDS-12% PAGE. Visualization was achieved by staining with Coomassie brilliant blue. Lanes: 1, low molecular mass markers (Pharmacia Biotech); 2, induced cells (90 μg); 3, extract after heat treatment (16 μg); 4, purified *Pab* RFC complex (58 μg) (see Materials and Methods). The *Pab* RFC-large and *Pab* RFC-small subunits migrated at the expected sizes in agreement with the expected mass of 55 kDa and 38 kDa, respectively.

known as RFC boxes II–VIII (Figure 2(a)). When the inteins were excised, *Pab* RFC-small was found to be 37% identical with the human RFC p40 subunit. *Pab* RFC-large showed the conserved RFC boxes observed in the large human RFC p140 subunit, but not all of the motifs are present in the *Pab* RFC-large subunit. An additional box (I), showing homology to prokaryotic DNA ligases and poly(ADP-ribose) polymerases,[39] is a BRCT module and is part of the N-terminal region of the human RFC 140, but not in the *Pab* RFC-large subunit (Figure 2(a)). The identifiable boxes[40] shared by the five RFC polypeptides contain three well-defined motifs: two of these boxes contain putative ATP-binding sequences (boxes III and V) known as Walker A (P-loop) and B motifs (DEAD box), respectively (Figure 2(b)). Also, the conserved p21-like PCNA-binding motif (QXX(h)XX(a)(a))[41] is present at the C-terminal part of the *Pab* RFC-large subunit.

Expression and subunits composition of the *Pab* RFC complex

Pab RFC-small and *Pab* RFC-large genes were cloned separately into a pET expression vector. The small subunit was expressed as a soluble product, but unfortunately the large subunit remained insoluble (data not shown). To overcome this problem, we decided to co-express both subunits into *Escherichia coli*. The expressed *Pab* RFC was found in the soluble extract and N-terminal sequencing confirmed the identity of the subunits; the partial NH_2-terminal sequences were, as expected: VRDMEEVREV, for *Pab* RFC-small; and PEVPWVEKYRP, for *Pab* RFC-large. Heat treatments and alternative different protocols were

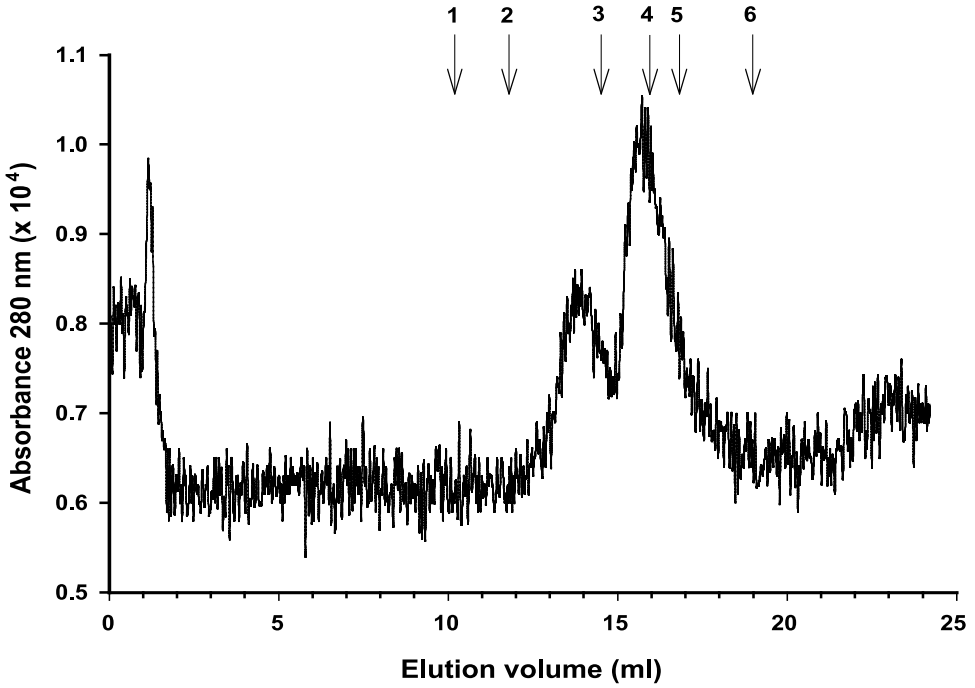


Figure 4. Analytical gel-permeation chromatography of the purified *Pab* RFC complex. *Pab* RFC was subjected to FPLC on a Superdex 200 HR column as described in Materials and Methods. The flow-rate was of 0.5 ml/minute and the molecular mass standards were: 1, thyroglobulin (669 kDa); 2, ferritin (440 kDa); 3, aldolase (158 kDa), 4, ribonuclease A (137 kDa), 5, bovine serum albumin (67 kDa); 6, chicken ovalbumin (43 kDa). The experiment was made with the *Pab* RFC complex purified by protocol A (see Materials and Methods).

used to purify the complex to apparent homogeneity (Figure 3, and see Materials and Methods). The protocol yielded large amounts of recombinant proteins, since typically about 5 mg of purified *Pab* RFC complex was obtained from 1.5 liters of an *E. coli* culture. To try to determine the subunit composition of the recombinant *Pab* RFC complex, different amounts of purified *Pab* RFC complex were separated by SDS-10% PAGE and stained with Coomassie brilliant blue. The gels were scanned and the relative concentrations of each subunit present in the purified *Pab* RFC fraction were quantified. *Pab* RFC complex yielded distinct mass ratios from 2:1 to 4:1, for *Pab* RFC-small and *Pab* RFC-large, respectively, depending on the preparation and the purification procedures. Analytical gel-filtration gave two peaks (Figure 4). The identity of the two RFC subunits was visualized by SDS-PAGE and silver staining. The two peaks eluted at 250 kDa and 134 kDa, corresponding to a putative heterohexamer (four RFC-small:two RFC-large) and a heterotrimer (two RFC-small:one RFC-large) complex.

The anti-hRFC p140 PCNA-binding domain antibody cross-reacts with *Pab* RFC-large

The *Pab* RFC-large fraction was subjected to Western blot analysis with either the mouse monoclonal anti-DNA-binding domain or the rabbit polyclonal anti-PCNA-binding domain antibodies generated from human RFC p140. The anti-PCNA-binding domain polyclonal antibody cross-reacted with *Pab* RFC-large (Figure 5). This further confirms the conclusions inferred from the protein sequence analysis, suggesting that both eukaryotic and archaeal large subunits share a common PCNA-binding motif (see below). In contrast, the absence of a structurally conserved DNA-binding domain within the *Pab* RFC-large, as expected by amino acid alignments, was confirmed by Western blotting (Figure 5).

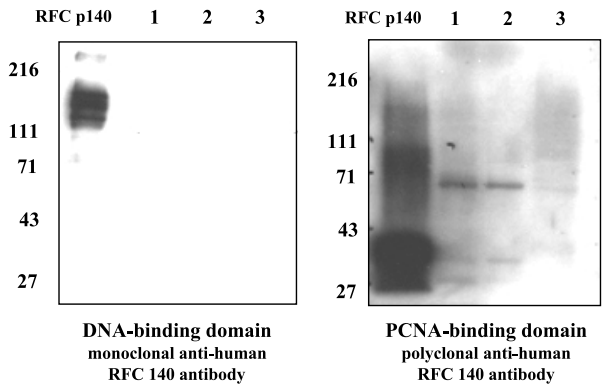


Figure 5. Anti-hRFC p140 PCNA-binding domain antibody cross-reacts with the *Pab* RFC-large subunit. The soluble and insoluble fractions of the recombinant *Pab* RFC-large were analyzed by SDS-PAGE and the gels were subsequently subjected to immunoblot experiments using antibodies against either 240 ng of the DNA-binding domain (left) or 437.5 ng of the PCNA-binding domains (right) of hRFC p140 (see also Figure 2). The positive controls used were the full-length recombinant hRFC p140 protein (left) and the recombinant PCNA-binding domain of hRFC p140 protein (right). Lane 1, crude pellet; lane 2, crude supernatant; lane 3, uninduced cells (negative control) (*E. coli* crude extracts: 114 μg). Sizes are indicated (in kDa) on the left.

Characterization of the *Pab* RFC-complex ATPase activity

The recombinant *Pab* RFC complex possesses a strong intrinsic ATPase activity. Unexpectedly, this activity was not stimulated by the presence of DNA (Figure 6(a)). Primed single-stranded circular templates or single-stranded linear templates, and double-stranded linear templates did not stimulate the ATPase activity of *Pab* RFC (results not shown). We initially hypothesized that DNA stimulation could be masked by the strong intrinsic *Pab* RFC ATPase activity. Therefore, a small amount of RFC (50 ng) was used for DNA titration. Again, DNA had no effect on ATPase activity, even at non-physiological concentrations (up to 150 μM; results not shown). Furthermore, treatment with polymin P and quantification of DNA were done to exclude unspecific DNA-stimulation on the *Pab* RFC ATPase activity, due to the presence of genomic DNA in our preparation. Finally, we excluded the possibility that DNA was denatured in the reaction mixtures. All experiments were performed at a temperature that was 5–10 deg. C lower than the melting temperature of the heteroduplex, preventing the destructuration of the double-stranded DNA. Finally, the ATPase activity of the *Pab* RFC-small subunit was DNA-independent (Figure 6(b)). The *Pab* RFC complex hydrolyzed dATP at a rate about half that for ATP (results not shown).

Next, the effect of recombinant *Pab* PCNA on the rate of ATP hydrolysis was examined in the presence of a circular DNA heteroduplex. *Pab* PCNA stimulated the intrinsic ATPase activity of *Pab* RFC complex (Figure 6(c)); 50 ng of *Pab* PCNA led to a twofold increase of the ATPase activity, either with 50 ng or 250 ng of *Pab* RFC complex. Interestingly, this effect was strictly DNA-dependent. Conversely, *Pab* PCNA did not stimulate the ATPase activity of the PabRFC-small subunit, in the presence or in the absence of the DNA heteroduplex (Figure 6(d)).

As shown in Figures 2(a) and 5, the PCNA-binding domain of the *Pab* RFC-large subunit shares conserved binding motifs in archaea and human. We investigated the effect of the human PCNA on the *Pab* RFC-complex ATPase activity. Since the hPCNA is not active above 50 °C, and the *Pab* RFC ATPase activity cannot be detected easily at 37 °C, we used 45 °C as the incubation temperature. In this heterologous system, higher protein concentrations were required to detect the effect, but the stimulation of the *Pab* RFC ATPase activity by the

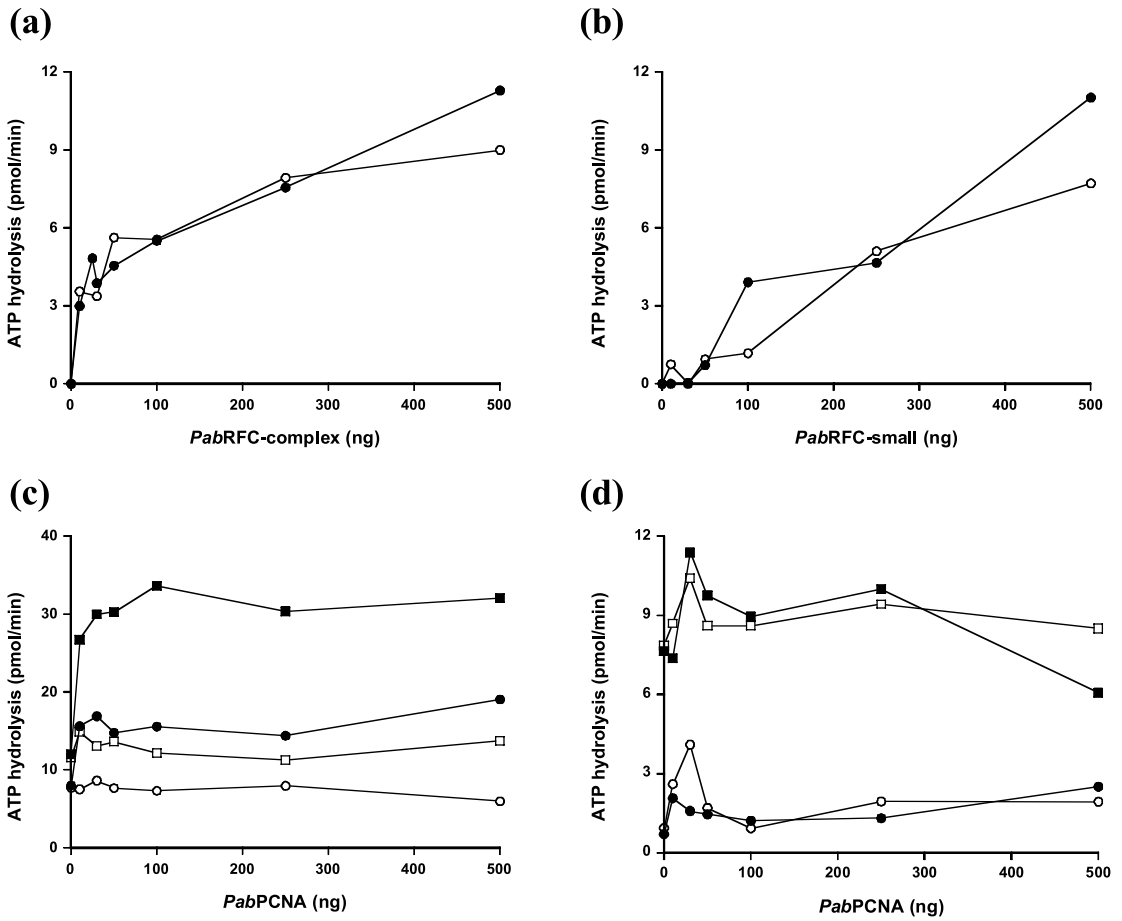


Figure 6. Characterization of the ATPase activity of recombinant *Pab* RFC. ATPase assays were performed with 15 μM (nucleotide concentration) of the circular M13ssc DNA heteroduplex as described in Materials and Methods. (a) DNA-independent ATPase activity of the *Pab* RFC complex. (b) DNA-independent ATPase activity of *Pab* RFC-small. Reactions were carried out either in the absence (open symbols) or in the presence (filled symbols) of the DNA heteroduplex. (c) DNA-dependent stimulation of *Pab* RFC complex ATPase activity by *Pab* PCNA. (d) DNA- and PCNA-independent ATPase activity of *Pab* RFC-small; *Pab* RFC and *Pab* RFC-small amounts were 50 ng (circles) or 250 ng (squares), in the absence (open symbols) or in the presence (filled symbols) of the DNA heteroduplex. Experiments were made with *Pab* RFC complexes purified with protocols A and B (see Materials and Methods).

hPCNA was evident and was strictly dependent on the presence of a DNA heteroduplex (Figure 7(a)). The maximal effect was obtained with 500 ng of hPCNA and with 500 ng of *Pab* RFC complex (Figure 7(b)).

Pab RFC is required for efficient DNA synthesis by *Pab* Pol II

When *Pab* PCNA and *Pab* RFC were absent, no full-length M13 product was detected (Figure 8, lanes 1 and 2). A major product was obtained near 1.5 kb, probably because of the presence of secondary structures within the circular M13 single-stranded DNA. *Pab* RFC complex stimulated the *Pab* PCNA-dependent DNA synthesis by *Pab* Pol II, increasing the amount of full-length M13 products (Figure 8, lanes 7 and 8). *Pab* PCNA alone stimulated *Pab* Pol II (Figure 8, lane 3), but to a much lesser extent than when *Pab* RFC complex was added (Figure 8, lane 8). ATP was not required to obtain full stimulation (compare lanes 7 and 8 of Figure 8). In fact, ATP seemed to lower the effects of the *Pab* PCNA (compare lanes 3 and 5 of Figure 8).

Pab RFC can load hPCNA on primed single-stranded circular DNA in the presence of non-hydrolyzable ATP

As the PCNA-binding domain is conserved in the *Pab* RFC, and hPCNA could stimulate *Pab* RFC ATPase activity in a DNA-dependent manner, we used the well characterized phosphorylable ph-PCNA derivative to measure the clamp-loading activity of the *Pab* RFC complex, using the cross-linked PCNA–DNA complex technique.[42] When ATP was added to the incubation mixture, no loading could be detected (Figure 9, lane 2). However, weak loading was detected in the absence of ATP (Figure 9, lane 4). This loading was not detected when double-stranded DNA was used, showing

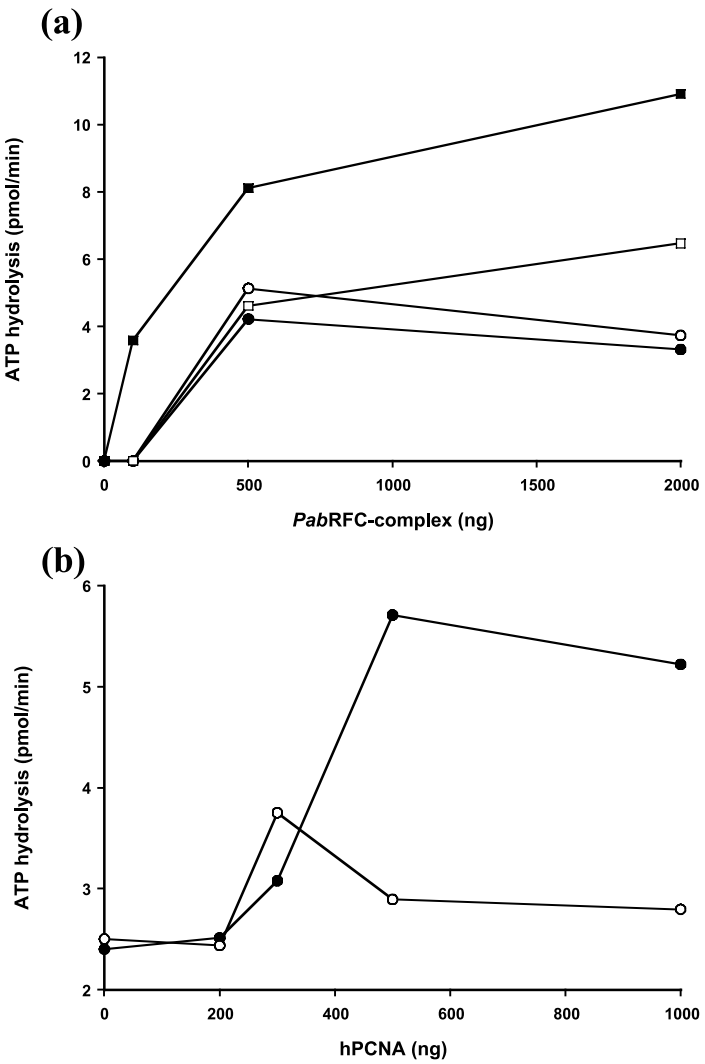


Figure 7. Stimulation of *Pab* RFC ATPase activity by hPCNA is dependent on DNA. The experimental conditions were as described in the legend to Figure 6 and in Materials and Methods, excepted that the incubation temperature was 45 °C. (a) The incubations were performed in the absence (open symbols) or in the presence (filled symbols) of primed M13ssc, either in the absence (circles) or in the presence (squares) of hPCNA (12.5 ng/μl). (b) Stimulation of the *Pab* RFC ATPase activity by hPCNA is dependent on DNA. Open circles, no DNA; filled circles, primed M13ssc. *Pab* RFC was 25 ng/μl. The *Pab* RFC complex was purified by using protocol A (see Materials and Methods).

that it is specific (Figure 9, lane 3). As ATP can stimulate the unloading activity of RFC, we next measured the effect of adenosine 5′-*O*-3-thiotriphosphate (ATPγS), a non-hydrolyzable ATP analogue, which stimulates only the loading activity of RFC.[43] An extensive loading was detected with this ATP analogue (Figure 9, lane 5), suggesting that ATP hydrolysis is not a prerequisite for loading but is likely involved in the clamp-unloading mechanism.

Discussion

We describe the isolation in large amounts and the biochemical characterization of the recombinant non-tagged dimeric RFC complex of the euryarchaeote *P. abyssi*. The two subunits are arranged in tandem within a 80 kb region that contains the origin of replication.[38] Other proteins involved in the initiation and elongation of DNA replication are present in this region (e.g. Cdc6, Pol II and Rad51). In bacteria, clusters of replication genes near the origin of replication trigger the effective assembly of replication forks at the origin.[44] The flanking region upstream of the RFC genes cluster contains a hexanucleotide AT-rich sequence (TATAAT) similar to the archaeal TATA box.[45] This sequence is located 15 nucleotides upstream of a TGGT motif. These putative promoter elements are equivalent to the eukaryotic regulation transcription boxes A and B, respectively.[46] Moreover, neither putative termination sequences nor putative transcription initiation boxes have been identified immediately downstream of the *Pab* RFC-small gene. This suggests that this gene cluster, organized as an operon, is co-transcribed like a polycistronic mRNA. This, in turn, probably reflects the functionality of a typical thymidine-rich archaeal termination sequence downstream of the *Pab* RFC-large gene (Figure 1).[47] Two putative translation signals have been identified upstream of the RFC genes. These regions are homologous to Shine–Dalgarno regulatory sequences,[47] and they most likely function as ribosome-binding sites.

The two RFC homologues share sequence homologies with their eukaryotic counterparts.

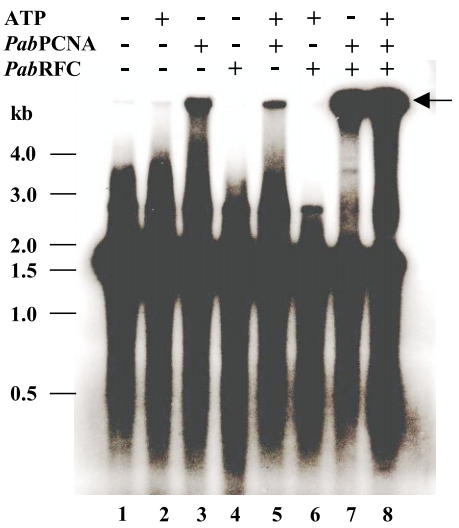


Figure 8. Effect of *Pab* RFC on the *Pab* PCNA-dependent primer-extension by *Pab* Pol II. One unit of *Pab* Pol II was incubated, as indicated in Materials and Methods, with 100 ng of *Pab* PCNA and 500 ng of *Pab* RFC. The reaction mixture was incubated at 65 °C for 30 minutes and subjected to agarose gel electrophoresis and autoradiography. The arrow indicates the position of the full-length M13 DNA. *Pab* RFC complexes purified by protocols A and B (see Materials and Methods) were used.

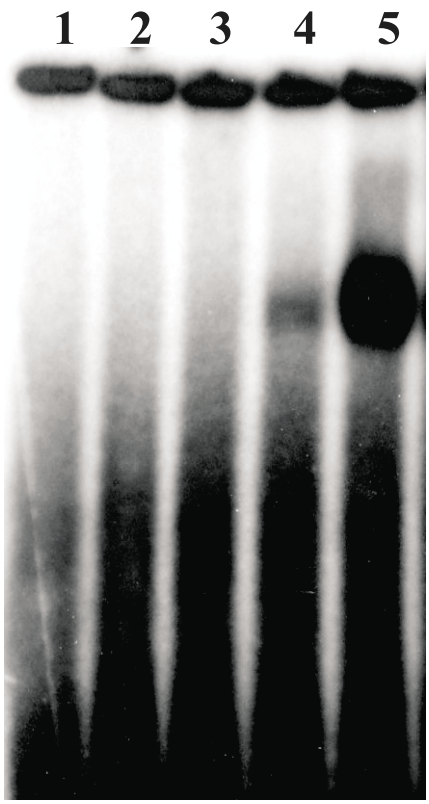


Figure 9. *Pab* RFC can load hPCNA onto a primed circular DNA in the presence of non-hydrolyzable ATP. The reaction conditions were as outlined in Materials and Methods. The reaction mixture (25 μl) contained 1 mM ATP or ATPγS, 500 ng of [^{32}P]ph-PCNA and 500 ng of *Pab* RFC and DNA: lane 1, M13ssc + ATP (control without *Pab* RFC); lane 2, M13ssc + ATP and *Pab* RFC; lane 3, M13ds, no ATP and *Pab* RFC; lane 4, M13ssc, no ATP and *Pab* RFC; lane 5, M13ssc + ATPγS and *Pab* RFC. The *Pab* RFC complex used was purified by protocol A (see Materials and Methods).

The Walker A motif is assumed to form a phosphate-binding loop that interacts with the triphosphate tail of ATP, whereas the Walker B motif interacts with the Mg^{2+} required for ATP hydrolysis.[48] The C-terminal part of both *Pab* RFC subunits is unique, indicating that they have a specific and distinct role in the RFC complex as in the case for the eukaryotic counterparts.[49] The N-terminal part of the *Pab* RFC-large subunit lacks the motif that shows homologies with prokaryotic DNA ligases and poly(ADP-ribose) polymerases (the BRCT module).[39] The function of this RFC A box is unknown, since RFC substitution in human and yeast cells by an RFC lacking the ligase homology domain showed no phenotype linked to DNA replication, DNA damage, telomere length maintenance or intrachromosomal recombination.[50,51]

The primary translation products of several archaeal proteins involved in DNA replication contain inteins that are excised rapidly *in vivo*.[52] Some RFC small subunits from archaea contain inteins. *Methanocaldococcus jannaschii* RFC small subunit contains three inteins (prototype alleles Mja RFC1, Mja RFC2 and Mja RFC3) at the RFC-a, RFC-b and RFC-c insertion sites. In *P. abyssi*, only sites a and c contain inteins, while other Pyroccosus species contain only intein Mja RFC1. In *Methanopyrus kandleri*, only the Mja RFC2 allele is found. Most archaeal RFC-small subunits (including *M. thermoautotrophicum* and *S solfataricus*) do not contain inteins. Also, no biochemical characterization of the RFC inteins has been reported so far. Insertion of inteins occurs at specific and conserved motifs, such as the phosphate-binding loop (P-loop) involved in the binding of phosphate nucleotide groups.[53] Despite these observations, our current understanding of such splicing proteins, specifically in DNA replication proteins, is very limited. The excision of the inteins is a prerequisite for protein function.

Well-studied clamp-loaders include the gp44/62 complex from bacteriophage T4, RFC from eukaryotes (human and yeast RFC) and the γ-complex from *E. coli*.[2] All of these clamp-loaders consist of five-subunits. Recently, three archaeal RFC homologues were characterized: *Mth* RFC from *M. thermoautotrophicum*,[36] *Sso* RFC from *S. solfataricus*,[34] and *Pfu* RFC from *P. furiosus*.[35] As described for *Mth* RFC,[36] the stability of the *Pab* RFC complex is dependent on high salt concentration. Indeed, dialysis against a low ionic strength buffer led to the dissociation of the complex, releasing *Pab* RFC-small and *Pab* RFC-large. *Pab* RFC-large then formed an insoluble aggregate.

Table 1. Summary of the effects of DNA and PCNA on the ATPase activity of eukaryotic and archaeal RFC complexes

	Effect of DNA	Effect of PCNA		SSB protein	Reference/source
		−DNA	+DNA		
H. sapiens	Highly dependent	No effect	Large effect	Not required	12,65–67
S. cerevisiae	Highly dependent	No effect	Large effect	Not required	6,68
M. thermoautotrophicum	Highly dependent	No effect	Large effect[a]	Required	36
S. solfataricus	Highly dependent	No effect	No effect	Not tested	34
P. furiosus	Moderately dependent	Inhibition	Large effect	Not tested	35
P. abyssi	Not dependent	No effect	Large effect	Not tested	This study

[a] Reverses the inhibition by the RPA protein.

Also, *E. coli*-expressed *Pab* RFC-large was completely insoluble. Different results were reported on the respective subunit composition. The large and small subunits of the *M. thermoautotrophicum* clamp-loader assemble in a hexameric structure, containing two large and four small subunits. The recombinant *S. solfataricus* RFC described to be ATP-dependent, is organized into a heteropentamer with one large and four small subunits.[34] The *Pfu* RFC complex consisted of three or four small subunits and one or two large subunits,[35] but further biochemical and structural analyses are apparently necessary to characterize the active form of the *Pfu* RFC complex. Indeed, the gel-filtration and glycerol gradient-centrifugation protocols, which have been described to determine the oligomeric structure of archaeal RFC complexes, cannot distinguish definitively between a pentamer and a hexamer. In this study, gel-filtration chromatography yielded two species, probably as a result of a partial dissociation of the *Pab* RFC complex on the column at the low concentration of salt used. Sedimentation equilibrium would be the most appropriate technique if a precision better than 10% could be achieved and if the structure of the RFC complex would be symmetrical. Recently, the atomic structure of the clamp-loader small subunit from *P. furiosus* was reported.[54] This complex assembled as a double trimer. This organization may reflect an alternative conformation adopted by the clamp-loader when only a subset of subunits is present in the complex,[55] but could indicate that the structure of the RFC is asymmetric. Therefore, the resolution of the oligomeric structure of the archaeal RFC complex awaits the collection of crystallographic data. This question takes on particular importance, as substantial differences in clamp-loading mechanisms could be associated with different RFC subunits complexes. Because subunits contain a consensus ATP-binding domain, the relative numbers of ATP molecules localized either in unique or identical subunits could differ.[56] This is important, given that multiple stepwise ATP-binding events are required to load PCNA onto primed DNA,[7] and that the ATP-binding domains of the small RFC subunits are essential for DNA recognition and clamp-loading.[8] In addition, the eukaryotic small subunits core complex may have different specific functions upon association with different types of large subunits.[57,58]

The ATPase activity of the archaeal RFC differs from that of the eukaryotic RFCs (Table 1) and there are remarkable differences between the four archaeal RFC complexes. The ATPase activity of *Pab* RFC is strongly DNA-independent. Similar results were obtained with RFC fraction A and the polymin P-treated RFC fraction B, which excluded any DNA from the preparation of the *Pab* RFC. The *Pab* RFC-small subunit had similar ATPase activity compared to that of the RFC complex. Moreover, ATPase activity assays were performed in the presence of varying amounts of poly(dA)/oligo(dT), primed M13ssc or primed λ DNA, and no stimulation was observed with any concentration of DNA tested (data not shown). The effect of PCNA was strictly DNA-dependent. The ATPase activity of the *P. furiosus* RFC complex was activated only moderately by DNA,[35] which would suggest that a low sensitivity of the RFC to DNA is a general feature within the Thermococcales group. The effect of PCNA on the *Mth* RFC ATPase activity was strictly dependent on the presence of SSB.[36] This may be the case for the *Sso* PCNA, as the measurements were made in the absence of SSB.[34] The *Pab* RFC complex behaves like a DNA-dependent PCNA-activated ATPase. This high DNA-dependency of the effect of PCNA would indicate that the *Pab* RFC complex preparation is not contaminated by DNA.

The RFC homologue from *P. furiosus* is not strictly dependent on ATP for its clamp-loading activity.[35] It has been suggested that *Pfu* RFC possesses the ability to unload the PCNA from the DNA heteroduplex and that dATP could substitute for ATP in the loading reaction. However, earlier studies looked for dATPase activity or DNA polymerase stimulation. In this study, we measured the loading that could take place in the absence of any hydrolyzable ATP analogue. Therefore, we suggest that ATP hydrolysis is not a prerequisite for loading but might enhance the clamp-unloading activity of the *Pab* RFC complex. These observations, and the fact that archaeal PCNA can stimulate DNA polymerase on primed circular DNA, in the absence of RFC, are still puzzling. An explanation could be that PCNA can perform subunit exchange at elevated temperatures (60–65 °C)

and thus could be loaded onto circular DNA, in the absence of RFC. This model, already suggested for different archaeal DNA replication systems, suggests a potential new role of archaeal RFC. Our findings strongly support this idea: (i) PCNA can be loaded spontaneously without the clamp-loader; (ii) ATP hydrolysis is not a prerequisite for loading; (iii) the spontaneously loaded PCNA, together with DNA, clearly stimulates the ATPase activity of the RFC. Then, the question arises: what is the enzymatic step that requires ATP hydrolysis? We propose that a new functional role of RFC could be attributed to its clamp-unloading activity. Other arguments favor this hypothesis. PCNA stimulation of the processivity of DNA pol II was optimal either with or without RFC, whereas in the presence of both replication factors (RFC and PCNA) and ATP, the distributivity of the polymerase was stimulated (increasing amounts of intermediates). Thus, the altered polymerase activity might be due to the clamp-unloading RFC dynamic process.

We showed that the PCNA-binding domain is structurally and functionally conserved from archaea to human. The fact that the archaeal PCNA can stimulate the activity of DNA polymerase δ,[29] and that human RFC interacts functionally with the archaeal PCNA,[59] emphasizes that the structure of the PCNA is conserved. We have demonstrated that the archaeal RFC interacts functionally with the hPCNA, and can load the sliding clamp onto singly primed, single-stranded circular DNA. The PCNA-binding domain is conserved among all the RFC subunits. However, the PCNA-binding domain of the human RFC p140 was sufficient to load the PCNA onto DNA.[50] On the other hand, the RFC small subunit complex of *P. furiosus* was able to stimulate the PCNA-dependent primer-extension abilities of the cognate DNA polymerase.[60] Therefore, there could exist more than one site of interaction between the clamp-loader and the clamp. A conserved PCNA-binding motif found in a variety of proteins interacting with PCNA[41] was reported at the C terminus of the *Archaeoglobus fulgidus* RFC large subunit.[61] Interestingly, this motif is present in all euryarchaeota (protein sequences available for 11 species), but is lacking in crenarchaeota with the exception of *Aeropyrum pernix* (four protein sequences available). The eukaryotic RFC could have evolved from a hypothetical ancestral homo-hexameric clamp-loader and the archaeal RFC would represent an intermediate stage of this evolution. Therefore, one can expect that studies on the DNA replication machinery of archaea will provide important clues for understanding the complex mechanisms of DNA replication in eukaryotes.

Materials and Methods

Materials

We used the *P. abyssi* Orsay strain, from which the total genome sequencing was performed by Genoscope (Evry, France). *E. coli* DH5α and HMS174(DE3) were purchased from Clontech Laboratories (Basingstoke, UK) and Novagen (Madison, WI), respectively. Deoxyribonucleoside 5′-triphosphates, [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Saclay, France). Polyacrylamide gel electrophoresis (PAGE) reagents were purchased from BioRad, Ivry sur Seine, France. Source Q was obtained from Amersham Pharmacia Biotech and hydroxyapatite "high resolution" was purchased from Fluka (Buchs, Switzerland). All other chemicals were molecular biology grade reagents from Sigma-Aldrich (St. Louis, USA).

Enzymes and proteins

Phage T4 DNA ligase, modification enzymes and restriction enzymes were obtained from Roche-Diagnostics (Mannheim, Germany). The phosphorylable derivative of human PCNA (ph-PCNA) was obtained as described.[42] The modified PCNA contains 18 additional amino acid residues at the C terminus, which generate a phosphorylation site for heart muscle protein kinases. The catalytic subunit of the non-cyclic AMP-dependent protein kinase from bovine heart muscle was purchased from Sigma-Aldrich.

Nucleic acids

Single-stranded circular (ssc) M13mp18 DNA was obtained from Amersham Pharmacia Biotech. Singly primed M13mp18ssc was prepared by incubating 125 μl of M13mp18ssc (0.2 mg/ml) with 2 μl of a complementary oligonucleotide primer (M13prim) (5′-ATTCGTAATCATGGTCATAGCTGTTTCCTG-3′) (1 mg/ml) and 125 μl of annealing buffer: 20 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$, 250 mM NaCl. The reaction mixture was heated at 75 °C for 15 minutes and then cooled slowly to room temperature.

Buffers

Buffer A: 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 2 mM β-mercaptoethanol, 10% (v/v) glycerol.

Buffer B: 50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol.

Buffer C: 25 mM potassium phosphate (pH 7.5), 1 mM DTT, 500 mM NaCl, 10% (v/v) glycerol.

Buffer D: 0.3 M potassium phosphate (pH 7.5), 1 mM DTT, 500 mM NaCl, 10% (v/v) glycerol.

Buffer E: 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 500 mM NaCl, 10% (v/v) glycerol.

Buffer F: 20 mM Tris–HCl (pH 6.8), 1 mM DTT, 0.1 mM EDTA, 10% (v/v) glycerol.

Buffer G: 10 mM Tris–HCl (pH 6.8), 1 mM DTT, 300 mM NaCl, 5% (v/v) glycerol.

Buffer H: 10 mM Tris–HCl (pH 6.8), 1 mM DTT, 1 M $MgCl_2$, 5% (v/v) glycerol.

Buffer I: 10 mM Tris–HCl (pH 6.8), 1 mM DTT, 50 mM NaCl, 5% (v/v) glycerol.

Buffer J: 10 mM Tris–HCl (pH 6.8), 1 mM DTT, 50 mM NaCl, 250 mM potassium phosphate (pH 6.8).

Computer analysis of protein sequences

Protein and gene sequences were retrieved from the NCBI Entrez sequence database. Multiple sequence alignments were produced using the ClustalW program.[62]

Cloning of the *P. abyssi* RFC genes

RFC-small encoding the small subunit (accession number PAB0068; NP 125803)

The inteins were removed by the splicing overlap extension strategy.[63] Briefly, internal and external oligonucleotide primers were designed to amplify the gene encoding the *Pab* RFC-small subunit from *P. abyssi*. External oligonucleotide primers RF1ini (forward) (5′-GGCCTCTAATTTCATATGCGTGACATGGAG-3′) and RF1end (reverse) (5′-ATGGGTCGGATCCTTATTTCTTCTTTCCGAT-3′) were designed to contain the *Nde* I and *Bam* HI restriction sites, respectively (underlined). The *Nde* I site contains the initiation codon ATG. Therefore, the Val that is given as the initiator amino acid in the annotated genome was mutated to Met. The *Bam* HI site is immediately after the stop codon. Internal oligonucleotide primers were designed to be completely homologous to the RFC-small gene: Int1-2 (reverse): 5′-AGGGCCAGAGCGGCGGTTGTCTTTCCAACTCCTGGGGGCC-3′; Int3-4 (forward): 5′-GGCCCCCAGGAGTTGGAAAGACAACCGCCGCTCTGGCCCT-3′; Int5-6 (reverse): 5′-ATCTTTGACGAATAGTTGCAGCTTAAAATAAACCTTACGT-3′; Int7-8 (forward): 5′-ACGTAAGGTTTATTTTAAGCTGCAACTATTCGTCAAAGAT-3′.

The entire RFC-small subunit (993 bp) was obtained by fragment complementation after three rounds of PCR (90 °C, one minute; 50 °C, one minute; 72 °C, one minute; ten cycles, DyNAzyme EXT DNA polymerase from Finnzymes, Espoo, Finland). The region was cloned and sequenced by the dideoxy chain termination method.

The PCR-amplified RFC-small subunit was digested with *Nde* I and *Bam* HI, and ligated into the *Nde* I-*Bam* HI sites of linearized pET11a *E. coli* ampicillin-resistant vector. The final product was called pRFC-small.

RFC-large encoding the large subunit (accession number PAB0069; NP 125804)

The RFC-large gene from *P. abyssi* was amplified from genomic DNA. The PCR-primers were as follows: RF2ini (forward) (5′-ATAAAGTGATGACATATGCCAGAAGTTCCC-3′) and RF2end (reverse) (5′-GGCCCTACTGGATCCAGCTACTTCTTTATG-3′). These primers contained *Nde* I and *Bam* HI restriction sites (underlined), respectively. The PCR product (1440 bp) was cloned into the *Nde* I-*Bam* HI sites of linearized pET26b(+) *E. coli* kanamycin-resistant vector. The final product was called pRFC-large. The integrity of the insert was confirmed by DNA sequencing.

Production and purification of recombinant *Pab* RFC proteins

Both pRFC-small and pRFC-large constructs containing the bacteriophage T7 gene promoter and leader sequence were introduced into *E. coli* strain MC1061(DE3) provided by J. M. Masson (IBPS, Toulouse, France). *E. coli* MC1061(DE3) harboring pRFC-small and pRFC-large were grown at 37 °C in 3 liters of LB medium with ampicillin (100 μg/ml) and kanamycin (30 μg/ml). The cells were grown to an $A_{600\ nm}$ of 0.8 and then IPTG was added to a final concentration of 0.6 mM to induce the overproduction of the recombinant proteins. The cells were cultured for a further four hours with gentle shaking (150 rpm). Then, they were centrifuged and the pellet was resuspended in 50 ml of buffer A. Bacterial lysates were prepared by sonication (30 × two second pulses then 2 × 30 second pulses). Cellular debris were removed by centrifugation at 15,000*g* for 45 minutes at 4 °C, and the pellet was resuspended in 20 ml of buffer A, and subjected to a second round of sonication. After centrifugation, the two supernatants were pooled (65 ml; protein concentration: 4.2 mg/ml) and heated at 75 °C for 15 minutes, followed by centrifugation (15,000*g* for 45 minutes at 4 °C). The supernatant (59 ml; protein concentration: 1 mg/ml) was heated for ten minutes at 80 °C and clarified by centrifugation for 45 minutes at 15,000*g*. The supernatant (55 ml; protein concentration: 1 mg/ml) was divided into two equal fractions, A and B, and subjected to two different purification protocols as outlined below:

Fraction A (27.5 ml) was put through an anion-exchange column (Source Q, 16 mm × 7.5 cm) (1 ml/minute) pre-equilibrated with buffer B. The column was washed with 80 ml of buffer B and the proteins were eluted with a 90 ml linear gradient from buffer B to buffer B containing 1 M NaCl. Fractions (2 ml) were collected and analyzed by SDS-PAGE. The fractions containing the RFC complex (centered at 0.2 M NaCl) were pooled (6.5 ml; protein concentration: 0.7 mg/ml) and loaded onto a hydroxyapatite column (0.5 ml/minute) (16 mm × 9.5 cm) pre-equilibrated with buffer C. The column was washed with 40 ml of buffer C and proteins were eluted with a 70 ml linear gradient from buffer C to buffer D (2 ml fractions). Fractions that contained the pure recombinant protein (15 ml; protein concentration: 0.4 mg/ml; centered at 180 mM potassium phosphate) were pooled.

Fraction B (27.5 ml) was adjusted to 1 M NaCl by adding 50 mM Tris–HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 4 M NaCl, 10% (v/v) glycerol, and nucleic acids were precipitated by adding polymin P to a final concentration of 0.2% (w/v), under gentle agitation for ten minutes at room temperature. After centrifugation (15,000*g* for 45 minutes), the supernatant was collected and solid ammonium sulfate was added over a period of one hour, at 0 °C, to obtain 80% saturation of ammonium sulfate. The mixture was then stirred for one hour and centrifuged at 15,000*g* for one hour. The resulting pellet was washed with 18 ml of 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 0.5 M NaCl, ammonium sulfate to 80% saturation, and centrifuged (15,000*g* for one hour at 4 °C). The pellet was resuspended in 5 ml of buffer E and dialyzed twice against 1.5 liters of buffer E. The fraction was then subjected to phosphocellulose chromatography on a 26 mm × 12 cm column equilibrated with buffer E (0.5 ml/minute, 2 ml fractions). Fractions containing protein were pooled (20 ml; protein concentration: 0.5 mg/ml) and subjected to hydroxyapatite column chromatography as described for fraction A. Finally, the fraction containing the pure recombinant protein (13.5 ml; protein concentration: 0.4 mg/ml) were pooled.

Fractions A and B containing the RFC complex were concentrated by centrifugation on a Centriprep 10 device (Millipore, Molsheim, France), and stored at −80 °C after dialysis against 20 mM Tris–HCl (pH 7.5), 2 mM DTT, 0.5 mM EDTA, 0.5 M NaCl, 10% (v/v) glycerol. The final yield of recombinant proteins purified by these

two protocols was about 3–5 mg per liter of bacterial culture.

Production and purification of the recombinant *Pab* RFC-small subunit

E. coli HMS174(DE3) containing the pRFC-small construct was grown to an $A_{600\ nm}$ of 0.6 at 37 °C in 1.5 liters of 2 × YT broth with ampicillin (100 μg/ml). The overproduction of the recombinant protein was induced by adding IPTG to a final concentration of 1 mM. The cells were cultured for a further 3.5 hours and pelleted by centrifugation. They were resuspended in 25 ml of buffer A and subjected to the sonication protocol as described for the RFC fraction A, including a second round of sonication with 10 ml of buffer A. After centrifugation (15,000*g* for 45 minutes), the supernatant (35 ml; protein concentration: 9.8 mg/ml) was heated at 75 °C for 15 minutes, followed by centrifugation (30 ml; protein concentration: 1.5 mg/ml) and at 80 °C for ten minutes (29 ml; protein concentration: 1.3 mg/ml). After chromatography on Source Q (9.3 ml; protein concentration: 0.6 mg/ml; centered at 0.1 M NaCl) and hydroxyapatite (3 ml; protein concentration: 0.4 mg/ml; centered at 100 mM potassium phosphate), the fractions containing the pure recombinant *Pab* RFC-small were pooled and concentrated as described for *Pab* RFC complex. The final yield was about 0.8 mg per liter of bacterial culture.

Cloning the *P. abyssi* PCNA gene (*Pab* PCNA)

PCR was used to amplify the ORF encoding for the putative PCNA (accession number, PAB1465; NP 127054) from *P. abyssi*. Two primers were designed to introduce a *Nde* I site at the initiating methionine and a *Bgl* II site just next to the stop codon, respectively (underlined) PCNAini (forward): 5′-AGGTGCAAACATATGCCATTCGAGATAGTC-3′; PCNAend (reverse): 5′-AGTTAAAGATCTTTACTCCTCAACCCTGGG-3′.

The PCR product was digested with *Nde* I and *Bgl* II and ligated into the *Nde* I-*Bam* HI site of the *E. coli* expression vector pET26b(+) containing the bacteriophage T7 gene promoter and leader sequence. The identity of the gene was confirmed by nucleotide sequencing and the recombinant plasmid was designated p*Pab* PCNA.

Production and purification of the recombinant *Pab* PCNA

Recombinant *E. coli* HMS174(DE3) containing p*Pab* PCNA were grown to an $A_{600\ nm}$ of 0.6 in 1.5 liters of LB medium containing kanamycin (30 μg/ml) with gentle shaking (150 rpm). A final concentration of 1 mM of IPTG was then added. Five hours after induction, the cells were harvested, resuspended in 25 ml of 50 mM Tris–HCl (pH 8.5), 0.1 mM EDTA, 2 mM β-mercaptoethanol, 0.1 M NaCl, 10% (v/v) glycerol, and lysed by sonication (ten × two second pulses then two × 30 second pulses). Cellular debris was removed by centrifugation at 15,000*g* for 45 minutes at 4 °C. *E. coli* proteins present in the supernatant (25 ml; protein concentration: 4.5 mg/ml) were partially eliminated by two heat treatment steps, as described for RFC, and the resulting supernatant was dialyzed against buffer F. The solution (30 ml; protein concentration: 0.6 mg/ml) was then passed through an anion-exchange column (1 ml/minute) (Source Q, 16 mm × 10 cm) pre-equilibrated with buffer F. The proteins were eluted with a 90 ml linear gradient of buffer F to buffer F containing 1 M NaCl (2 ml fractions). The fractions containing *Pab* PCNA (detected by SDS-PAGE) were pooled (12 ml; protein concentration: 0.7 mg/ml; centered at 0.4 M NaCl) and loaded onto an hydroxyapatite column (0.5 ml/minute) (16 mm × 9.5 cm) pre-equilibrated with buffer G. The column was washed with 40 ml of buffer G, followed by 40 ml of buffer H, and finally the *Pab* PCNA was eluted with a 60 ml linear gradient from buffer I to buffer J. Fractions (1 ml) were collected. The fractions containing the *Pab* PCNA (10.5 ml; protein concentration: 0.14 mg/ml; centered at 0.2 M potassium phosphate) were concentrated, dialyzed against 50 mM Tris–HCl, (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 50% (v/v) glycerol and stored at −20 °C. The final yield was about 1 mg per liter of bacterial culture.

Terminal sequence analysis

The RFC subunits were subjected to electrophoresis in SDS-12% polyacrylamide gels, then transferred onto polyvinylidene difluoride (PVDF) membranes. Finally, the sequences of the N termini of both RFC subunits were determined.

Western blotting

E. coli HMS174(DE3) harboring pRFC-large was grown to an $A_{600\ nm}$ of 0.4 in 50 ml of LB containing kanamycin (30 μg/ml) at 37 °C. IPTG was then added to the culture to a final concentration of 1 mM and growth was continued for four hours. Cells were harvested by centrifugation for 15 minutes at 6000 rpm. The pellet was resuspended in buffer A and the total extract was used for immunoblot analysis. The proteins were separated on SDS-10% PAGE and transferred to nitrocellulose membranes. The blots were saturated with blocking buffer containing 5% (w/v) skimmed milk in TBS-T (Tris-buffered saline: 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20) for 30 minutes. The blots were then incubated for two hours at room temperature with either a 1:5000 dilution of the rabbit polyclonal anti-human RFC PCNA-binding domain region antibody (produced at the animal facility in the Institute of Animal Breeding of the University of Zürich) or a 1:1000 dilution of the mouse monoclonal anti-human RFC DNA-binding domain region antibody[39] in TBS-T with 2% (w/v) skimmed milk. The membranes were washed three times with TBS-T, and then incubated for two hours at room temperature with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit antibody or 1:10,000 dilution of horseradish peroxidase-linked anti-mouse IgG in 2% (w/v) skimmed milk in TBS-T. The membranes were washed three times with TBS-T and the secondary antibody was detected by the enhanced chemiluminescence system (SuperSignal® West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Protein quantification

Protein concentrations were determined by the Bradford method,[64] using the Biorad reagent, and bovine serum albumin as the standard.

Analytical gel filtration

To estimate the molecular mass of the RFC complex, purified RFC fractions were subjected to gel-filtration analysis using an Amersham Pharmacia FPLC system. A Superdex-200 HR 10/30 column (Amersham Pharmacia) was pre-equilibrated with 50 mM Tris–HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 10% (v/v) glycerol. The flow-rate was 0.5 ml/minute. Thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), ribonuclease A (137 kDa), bovine serum albumin (67 kDa) and chicken ovalbumin (43 kDa) were used as molecular mass markers.

ATPase activity assays

The standard reaction mixture (20 μl) contained 20 mM Hepes–NaOH (pH 7.5), 2 mM DTT, 50 μg/ml of bovine serum albumin, 3 mM $MgCl_2$, 100 mM NaCl, 50 μM ATP, 4 pmol of [γ-^{32}P]ATP (specific activity, 5000 Ci/mmol), 15 μM primed M13ssc DNA (nucleotide concentration) and different amounts of recombinant proteins as indicated in the Figure legends. The reaction mixtures were incubated for 30 minutes at 60 °C and the reaction quenched on ice by adding 10 μl of 30 mM EDTA. A portion (2 μl) of the products was separated by polyethyleneimine–cellulose thin-layer chromatography (TLC) (Merck, Darmstadt, Germany) in 0.5 M LiCl, 1 M formic acid. The amount of inorganic phosphate produced was then quantified by scanning the autoradiography plates.

Agarose gel electrophoresis of ph-PCNA–*Pab* RFC–DNA complexes

The human PCNA derivative (ph-PCNA) was labeled by incubation with [γ-^{32}P]ATP and the bovine heart muscle protein kinase (0.15 unit/μl) as described.[42] The [^{32}P]ph-PCNA–*Pab* RFC–DNA complex was assembled in 20 mM Hepes–NaOH (pH 7.5), 2 mM DTT, 0.2 mg/ml of bovine serum albumin, 3 mM $MgCl_2$, 0.1 M NaCl, in the presence of 4 ng/μl of primed M13ssc heteroduplex (total volume 25 μl). Concentrations of *Pab* RFC and ph-PCNA were 20 ng/μl. *Pab* RFC was incubated at 65 °C for ten minutes and subsequently added to the reaction mixture, followed by incubation at 45 °C for three minutes. Then, 2.5 μl of 1% (w/v) glutaraldehyde was added and the mixture was further incubated at 37 °C for ten minutes. Finally, 0.8% agarose gel electrophoresis were performed, in the presence of 0.1% (w/v) SDS, as described.[42]

DNA synthesis studies with *Pab* DNA pol II

To determine the stimulation, by the *Pab* RFC, of the primer-extension abilities of the family D *Pab* DNA polymerase, one unit of *Pab* Pol II was incubated in 20 mM Bis–Tris (pH 6.5), 2 mM β-mercaptoethanol, 0.4 mg/ml of bovine serum albumin, 10 mM $MgCl_2$, 0.2 mM each of the dNTPs, 60 μCi/ml of [α-^{32}P]dCTP, 12.5 μg of primed M13ssc, 0.5 mM ATP, 100 ng of *Pab* PCNA and 500 ng of *Pab* RFC. The reaction mixture (50 μl) was incubated at 65 °C for 30 minutes. NaOH (30 mM) and EDTA (1 mM) were then added and the reaction mixture analyzed by denaturing 1% (w/v) agarose gel electrophoresis and autoradiography. One unit of pol II corresponds to 1 nmol of dNTP incorporated into acid-insoluble materials, at 65 °C in one minute using poly(dA)/oligo(dT) as substrate.

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