Identification of a second proliferating cell nuclear antigen in the human malarial pathogen *Plasmodium falciparum*

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Abstract

Proliferating cell nuclear antigen seems to exist as a single form in higher eukaryotic cells and plays multiple roles in nucleic acid metabolism. We have identified a second additional proliferating cell nuclear antigen (PfPCNA2) in *Plasmodium falciparum* on the basis of several lines of evidence. (1) PfPCNA2, consisting of 264 amino acid residues with a predicted molecular mass of 30.2 kDa, shares only 29% identity and 53% similarity with PfPCNA1 at the amino acid level. (2) Southern blot analyses revealed that the hybridisation pattern of the *Pfpcna2* gene is completely different from that of the *Pfpcna1* gene. (3) Chromosomal localisation studies showed that *Pfpcna2* is located on chromosome 12 while *Pfpcna1* is located on chromosome 13. Northern blot analyses revealed two different transcripts of *Pfpcna2*, one expressed in both asexual and sexual erythrocytic stages, while the other existed only in the sexual stage, implying that PfPCNA2 may play multiple roles in DNA metabolism in different stages of the parasite. Recombinant protein of PfPCNA2, overexpressed in *Escherichia coli*, has been purified to near homogeneity and shown to form an oligomer, probably a trimer, as revealed by a size-exclusion chromatography and a native gel electrophoresis, suggesting that PfPCNA2, like its higher eukaryotic counterparts, may serve as a sliding platform which is capable of interaction with diverse proteins and regulation of their activities. © 2002 Australian Association for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Malaria; Proliferating cell nuclear antigen; DNA replication; *Plasmodium falciparum*

1. Introduction

Malaria kills between one and three million humans, mostly children, every year, making it one of the leading causes of death from an infectious disease (Marshall, 2000). The lack of an effective vaccine and the development of *Plasmodium* resistance to many existing antimalarial drugs have aggravated the situation. It is therefore imperative that our understanding of the fundamental biology and biochemical processes at different stages of the parasite be improved, to facilitate the identification of new targets for the development of novel drugs and vaccines. DNA replication represents such a key process of the parasite. There are at least five distinct points in the parasite life cycle when DNA replication occurs (White and Kilbey, 1996), two of which take place in the human host, i.e. in the hepatocytes and in the erythrocytes, and the remainder occurs in the mosquito vector. The selective blocking of DNA synthesis in the parasite should inhibit both the disease and the parasite transmission.

DNA replication proceeds in two sequential stages which appear highly conserved in eukaryotes. At the initiation stage, the origin recognition complex (ORC) composed of six subunits (ORC1–ORC6) binds to replication origins during most or all of the cell cycle and serves as an initiator (Kelly and Brown, 2000). Cdc6/Cdc18 and Cdt1 function as loading factors which cooperate with ORC to recruit six different but related polypeptides known as the mini-chromosomal maintainence (MCM) proteins onto the origins (Kelly and Brown, 2000; Maiorano et al., 2000; Nashitani et al., 2000). MCM has DNA helicase activity that may be responsible for unwinding the double-stranded DNA (dsDNA) producing a single-stranded DNA (ssDNA) (Labib and Diffley, 2001). During the elongation stage, the ssDNA-binding protein, replication protein A (RPA), binds and stabilises ssDNA at the replication fork (Wold, 1997). DNA polymerase α (pol α)/primase, consisting of four subunits, then interacts with the RPA bound on ssDNA. The primase subunit of pol α/primase generates an RNA primer and the polymerase subunit adds a stretch of deoxyribonucleotides to the RNA primer (Waga and

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Stillman, 1998). Replication factor C (RFC), a complex of five subunits (RFC1–RFC5), gains access to the primer–template junction by binding the RPA and serves as a clamp loader (Mossi and Hübscher, 1998). Proliferating cell nuclear antigen (PCNA) then binds to the RFC–DNA complex in the presence of adenosine triphosphate (ATP) and forms a homotrimeric complex (Cox, 1997; Kelman, 1997). Next, DNA polymerase δ (pol δ) interacts with PCNA, which functions as a sliding clamp holding the polymerase on the primer terminus. The clamped DNA polymerase is highly processive, adding thousands of nucleotides to the $3'$ end of the RNA–DNA primer (Waga and Stillman, 1998). The initiator RNA primers are then removed by RNase H1 and Fen1 nucleases (Lieber, 1997). Finally, the daughter DNA fragments are sealed by DNA ligase I (Waga and Stillman, 1998). The process of DNA replication is highly coupled to cell cycle progression and to DNA repair to maintain genome integrity.

Although considerable knowledge of DNA replication in eukaryotes has been obtained from studies on the SV40 in vitro replication system, very little is known about DNA replication in the malaria parasite. To date, only two proteins associated with replication initiation and four components involved in DNA elongation have been identified and isolated from Plasmodium falciparum (White and Kilbey, 1996). These include MCM4 (Li and Cox, 2001), ORC1 (Li and Cox, 2002), PCNA (Kilbey et al., 1993), DNA primase (Prasartkaew et al., 1996), DNA pol α (Abu-Elheiga et al., 1990; Choi and Mikkelsen, 1991; White et al., 1993) and DNA pol δ (Fox and Bzik, 1991; Ridley et al., 1991). We are interested in DNA replication in P. falciparum. As a part of our efforts, in this paper we describe a second P. falciparum-proliferating cell nuclear antigen (PfPCNA2).

2. Materials and methods

2.1. Parasite and parasitic materials

Plasmodium falciparum clones T996 and 3D7A were used in this study. Parasite DNA and total RNA were extracted from cultures of P. falciparum 3D7A as described previously (Li et al., 1996). The first-strand cDNA was generated from 1 μg of total RNA using the random hexamer primer in the rapid-amplification-of-$5'$-cDNA-ends system of synthesis (Gibco) with DNase I (Li and Baker, 1997).

2.2. Oligonucleotides

Oligonucleotides PC1 ($5'$-CCTTGCACCAAAAATAG-GAGACTAC-$3'$, 1121–1145), PC2 ($5'$-ACCTAAAACG-CAATTTTTATCACACC-$3'$, 532–557), PC$_B$ ($5'$-CGGGATCCCATATGTTTGAATGCAGAATAG-$3'$, 240–258) containing BamHI and NdeI restriction sites and PC$_E$ ($5'$-CCCAAGCTTAGTAGTCTCCTATTTTTG-$3'$, 1129–1148) containing HindIII site were ordered from Genosys

and used for either Vectorette PCR (PC1 and PC2) to screen Vectorette libraries (Li et al., 1996) or construction of the expression vectors (PC$_B$ and PC$_E$) to produce recombinant proteins. The Vectorette primer was obtained from Cambridge Research Biochemicals.

2.3. Construction and screening of Vectorette libraries

Vectorette libraries were constructed from P. falciparum 3D7A genomic DNA as described previously (Li et al., 1996). With a specific primer and a universal Vectorette primer, PCR was used to screen Vectorette libraries (Li et al., 1996). PCR products were cloned into the pGEM-T vector (Promega) and sequenced using an ABI PRISM (model 377) automatic sequencing facility in this department.

2.4. Southern and Northern blot analyses

Approximately 4 μg of genomic DNA (clone 3D7A) digested with a number of restriction enzymes was used for Southern blotting and approximately 10 μg of total RNA extracted from both asexual and sexual erythrocytic stages of P. falciparum 3D7A was employed for Northern blotting (Li et al., 1996). The Hybond N $^+$ nylon membrane blots containing DNA or RNA were probed with a ^{32}P-labelled PC$_B$–PC$_E$ fragment as described previously (Li et al., 1996). Filters were washed at 56°C in $1 \times$ SSC/0.1% SDS for 40 min and then in $0.5 \times$ SSC/0.1% SDS for 30 min and autoradiographed at -80°C.

2.5. Pulse-field gel electrophoresis

Preparation of the agarose blocks containing chromosomal DNA, gel electrophoresis using a Bio-Rad CHEF DRII system, and treatment of the blotted membrane were carried out as described previously (Li and Baker, 1997). The blot was hybridised with the PC$_B$–PC$_E$ DNA probe and processed further as described above.

2.6. Recombinant protein expression and purification

The PC$_B$–PC$_E$ fragment of Pfpcna2 was amplified from cDNA and cloned into the pGEM-T vector. The resulting pGEM-T-PfPCNA2 construct was sequenced to verify the insert cDNA sequence. An NdeI–HindIII fragment was released from pGEM-T-PfPCNA2 and subcloned into either an NdeI/HindIII-digested pT7.7 vector or modified pET-11 vector (containing a histidine tag) to generate naked or His-tagged fusion protein in Escherichia coli. pET-11-PfPCNA2 encoding a 264 amino acid protein fused with MetHis8 at its N-terminus was transformed into E. coli [BL21-CodonPlus^{TM} (DE3)-RIL] competent cells. After induction with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) at 37°C for 4 h, the bacterial cells were collected by centrifugation, the pellet resuspended and sonicated in ice-cold 25 mM Tris–HCl (pH 7.4) containing 250 mM NaCl, 1 mM DTT, 0.1% Triton X-100, and a protease inhibitor mixture (Boehringer Mannheim). The lysate was
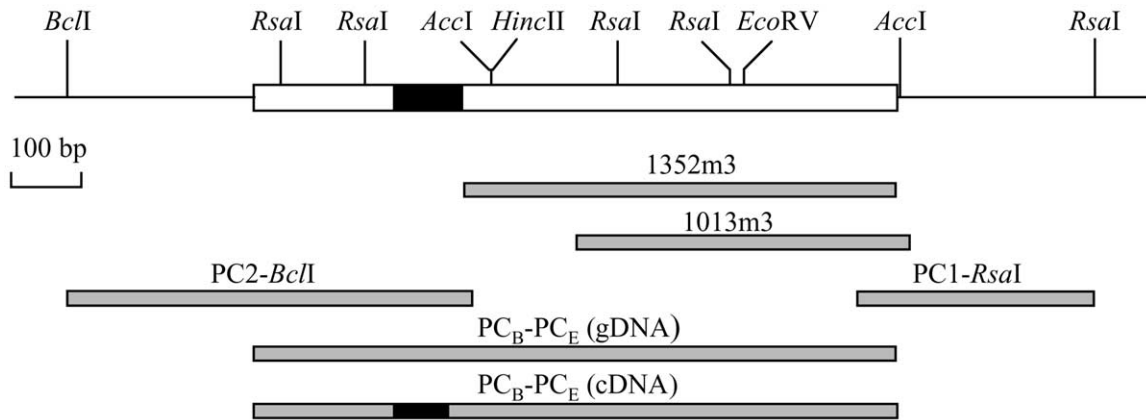
Fig. 1. A schematic representation of a partial restriction map of the *Pfpcna2* gene and the overlapping fragments used to determine its nucleotide sequence. The open boxes indicate the exon coding regions of the *Pfpcna2* gene and the black box represents the intron. The fragments of 1352m3 and 1013m3 were obtained from the *Plasmodium falciparum* tag database. The fragments of PC1-*RsaI* and PC2-*BclI* were derived from the Vectorette PCRs. PC$_B$–PC$_E$ (gDNA) and PC$_B$–PC$_E$ (cDNA) were amplified from genomic DNA and cDNA, respectively.

centrifuged at 40,000 g for 60 min at 4°C to remove solid particles. The resulting supernatant was subjected to nickel chelate affinity chromatography. The nickel column was equilibrated with five bed volumes of 15 mM imidazole in 50 mM Tris–HCl pH7.4, 250 mM NaCl. The protein sample was loaded onto the column and washed with 15 bed volumes of the same buffer containing 40 mM imidazole. Elution was performed with an imidazole gradient of 50–1000 mM in the same buffer applied over 10 bed volumes. Fractions of 1 ml were collected and dialysed against dialysis buffer consisting of 50 mM Tris–HCl pH7.4, 250 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM β-mercaptoethanol and stored in 25% glycerol at −70°C.

2.7. Western blotting

The protein extracts were subjected to SDS-PAGE on 12% gels followed by electrotransfer of the proteins to a nitrocellulose membrane (Hybond-C super, Amersham). PCNA was detected by a mouse monoclonal anti-histidine tag antibody (Sigma) or a rabbit polyclonal antibody (3009) raised to a synthetic peptide (15-mer) of the C-terminus of human PCNA. Immunoreactive proteins were visualised using ECL™ reagents (Amersham).

2.8. Size-exclusion chromatography

A fast protein liquid chromatography (FPLC) system was used to determine the size of PfPCNA2. Recombinant PfPCNA2 (4 μg) was loaded onto the 7 ml Superdex 200 column (Pharmacia) which was then washed with 13 ml buffer (50 mM Tris–HCl pH7.4, 250 mM NaCl, 1 mM DTT). Fractions were collected using an Akta FPLC system. Protein peaks were determined by absorption at 280 nm and by western blotting using the monoclonal anti-histidine tag antibody.

3. Results

3.1. Identification of the Pfpcna2 gene and characteristics of its deduced protein

Two DNA fragments (tags 1352m3 and 1013m3) were found in the *P. falciparum* database encoding an overlapping protein fragment with a high sequence homology to maize PCNA (López et al., 1995). To obtain the remainder of the gene, two specific primers, PC1 (walking in the 3′ direction) and PC2 (walking in the 5′ direction), were constructed on the basis of the 1352m3 sequence and used in PCR to screen Vectorette libraries (Li et al., 1996). Two fragments (PC1-*RsaI* and PC2-*BclI*) were obtained and sequenced. To confirm the sequence obtained from the overlapping fragments, an independent PCR fragment (PC$_B$–PC$_E$) was amplified from the parasite (3D7A) genomic DNA and sequenced on both strands (Fig. 1). The sequence derived from overlapping tag and PCR fragments consists of 1,461 bp and contains an intron that consists of 114 nucleotides and interrupts the coding region, starting with the conserved dinucleotides GT and terminating with AG. To verify the size and location of the presumed intron, reverse transcriptase (RT)-PCR (Li and Baker, 1997; Li et al., 2000) was performed using PC$_B$ and PC$_E$ primers (data not shown). Sequencing of the RT-PCR product confirmed the precise exon–intron boundaries.

The open reading frame resulting from the removal of the intron encodes a protein of 264 amino acids (Fig. 2A) with a predicted Mr of approximately 30.2 kDa and an isoelectric point 4.87. Database searches revealed that the amino acid sequence of the predicted protein shares 43–56% similarity and 19–36% identity with proteins in the PCNA family. The predicted protein shares only 53% similarity and 29% identity with the known *P. falciparum* PCNA (Kilbey et al., 1993) (hereafter called PFPCNA1). Accordingly, we designated our protein PfPCNA2. As shown in Fig. 2, PFPCNA2

A.

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                 βA1        αA1            βB1       βC1          βD1        βE1
HsPCNA     1  MFEARLVQGSILKKVLEALKDLINEACWDISSSGVNLQSMDSSHVSLVQLTLRSEGFDTY
ScPCNA     1  MLEAKFEEASLFKRIIDGFKDCVQLVNFQCKEDGIIAQAVDDSRVLLVSLEIGVEAFQEY
PfPCNA1    1  MLEAKLNNASILKKLFECIKDLVNDANVDADESGLKLQALDGNHVSLVSLHLLDSGFSHY
PfPCNA2    1  MFECRID-GQFFKKLFETLKDICTEVNLECDENGIKMQSMDCSHVSLVDLNIVSDFFQHY
              *:* ::   ::*::::: :**         :      *: *::*  :* ** * :   * *
                 βF1    αB1              βG1              βH1        βI1
HsPCNA    61  RCDRNLAMGVNLTSMSKILK-CAGNEDIITLRAEDNAD----TLALVFEAPNQ--EKVSD
ScPCNA    61  RCDHPVTLGMDLTSLSKILR-CGNNTDTLTLIADNTPD----SIILLFEDTKK--DRIAE
PfPCNA1   61  RCDRERVLGVNIASLNKVFKLCGAN-ESVVISSKDDED----NLNFVFENNKE--DKVTN
PfPCNA2   60  RCDKNCVLGISINFMLKILS-VIKEKSTVFLFKEDNENDAVLNIGIIDEEEQSSADDSLE
              ***:    :*:  :   : *::      :   :      : ::  *   :     :
                                     βA2       αA2         βB2       βC2
HsPCNA   114  YEMKLMDLDVEQLGIP--EQEYSCVVKMPSGEFARICRDLSHIGDAVVISCAKDGVKFSA
ScPCNA   114  YSLKLMDIDADFLKIE--ELQYDSTLSLPSSEFSKIVRDLSQLSDSINIMITKETIKFVA
PfPCNA1  114  FSLKLMSIELDSLNIPDCEEGFDAEVELSSKELTNIFRNLSEFSDTVFIEIDSNCIKFTT
PfPCNA2  119  IQVKLINAQKEHLEIP--QSEYHCQCTMKSKKFQEFTKYLNSIGDNVSISMKKDAMILST
              :**:   : : * *    :  :        : * ::  : *  : * : *    : : : :
                   βD2           βE2      βF2      αB2        βG2
HsPCNA   172  SGELGNGNIKLSQTSNVDKEEEAVTIEMNEPVQLTFALRYLNFFTKATPLSSTVTLSMSA
ScPCNA   172  DGDIGSGSVIIKPFVDMEHPETSIKLEMDQPVDLTFGAKYLLDIIKGSSLSDRVGIRLSS
PfPCNA1  174  KGIVGDAEVALKPRDSTSEDDIGVTIKSKKKIKQSFAIKYLNLFSKSNILADVVVLGLSD
PfPCNA2  177  TG----SDIKVTK--QFTNDMTDISITCTKSVSQEFATRYLVMFSRASSLSDEVLISLSP
              *      : :        : :      * :** :  :   *: * : :*
                βH2             βI2
HsPCNA   232  DVPLVVEYKIADM---------GHLKYYLAPKIEDEEGS--          261
ScPCNA   232  EAPALFQFDLKS----------GFLQFFLAPKFNDEE----          258
PfPCNA1  234  SRPIEFKYEIKDTSPDSDTLKIGFVKFFLAPKMDDDMDNKD          274
PfPCNA2  231  HIPISIKFNFKQQLTDLQDP--SHLTFFLAPKIGDY-----          264
              *   :: :              : ::****: *
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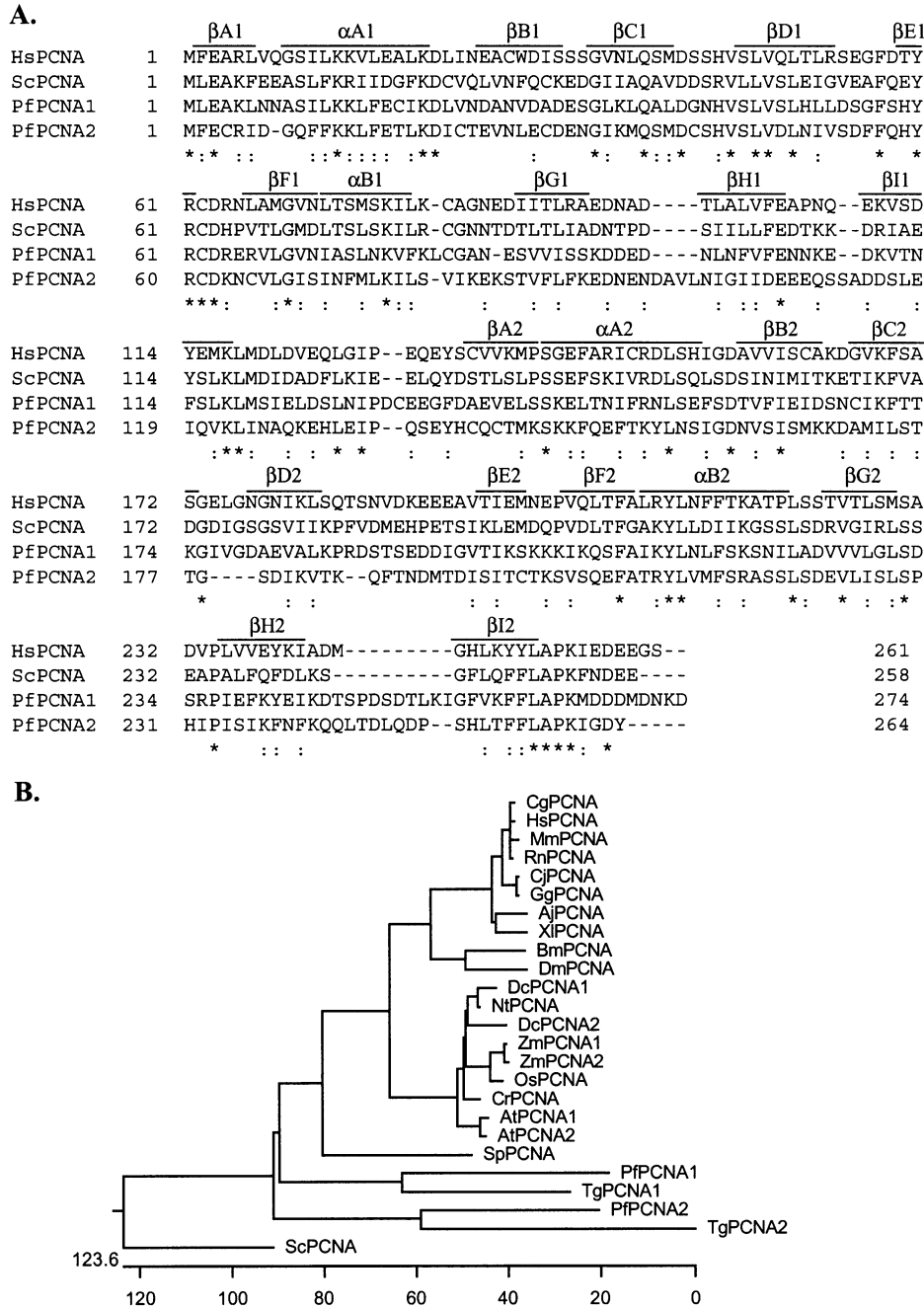
B.

Fig. 2. Characteristics of the PfPCNA2 protein. (A) Sequence alignment of *Saccharomyces cerevisiae*, human and *Plasmodium falciparum* PCNAs. The GenBank™/EMBL/DDJB database accession numbers are as follows: *S. cerevisiae* ScPCNA, P15873; *Homo sapiens* HsPCNA, P12004; *P. falciparum* PfPCNA1, P31008; and *P. falciparum* PfPCNA2, AF056205. Sequences were aligned with the CLUSTAL W (1.60) multiple sequence alignment programme. The amino acid residues are numbered to the left of the sequence. Identical residues are indicated with asterisks and conservative changes indicated with dots. The second structures based on the yeast PCNA (Krishna et al., 1994) are labelled on top of the sequence. (B) Phylogenetic comparisons of the eukaryotic PCNA proteins. The database accession number of each member is as follows: *Cricetulus griseus* CgPCNA, P57761; *Homo sapiens* HsPCNA, P12004; *Mus musculus* MmPCNA, P17918; *Rattus norvegicus* RnPCNA, P04961; *Coturnix japonica* CjPCNA, AJ301669; *Gallus gallus* GgPCNA, AB053163; *Anguilla japonica* AjPCNA, AB025357; *Xenopus laevis* XlPCNA, P18248; *Bombyx mori* BmPCNA O01377; *Drosophila melanogaster* DmPCNA, A34752; *Daucus carota* DcPCNA1, Q00268; *Nicotiana tabacum* NtPCNA, Q82793; *D. carota* DcPCNA2, Q00265; *Zea mays* ZmPCNA1, S52115; *Z. mays* ZmPCNA2, U87949; *Oryza sativa* OsPCNA, S14415; *Catharanthus roseus* CrPCNA, P24314; *Arabidopsis thaliana* AtPCNA1, Q9M7Q7; *A. thaliana* AtPCNA2, Q9ZW35; *Schizosaccharomyces pombe* SpPCNA, Q03392; *P. falciparum* PfPCNA1, P31008; *Toxoplasma gondii* TgPCNA1, AF242301; *P. falciparum* PfPCNA2, AF056205; *T. gondii* TgPCNA2, AF242302; and *S. cerevisiae* ScPCNA, P15873.
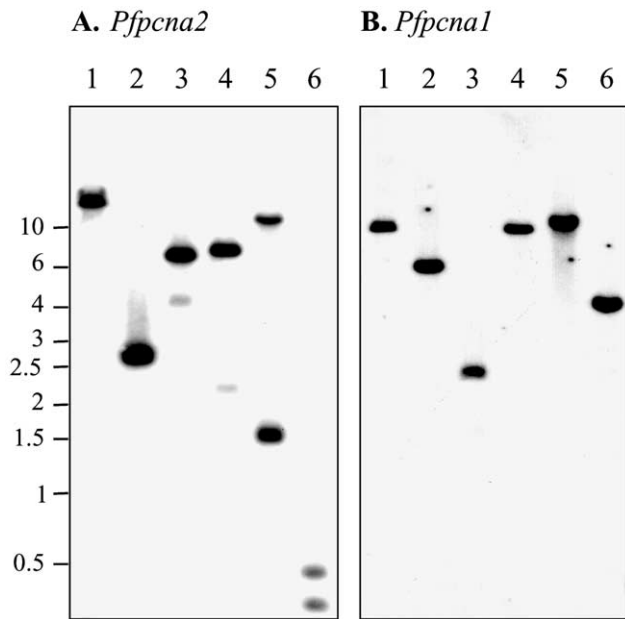
A. *Pfpcna2*   **B.** *Pfpcna1*

Fig. 3. Southern blot analysis of the *Pfpcna2* gene. Genomic DNA, 4 µg, from *Plasmodium falciparum* clone 3D7A was digested with restriction enzymes, electrophoresed on a 1.0% agarose gel, transferred onto a nylon membrane, and probed with (A) the *Pfpcna2* gene (the PC_B–PC_E fragment) and (B) the *Pfpcna1* gene, respectively. Lanes 1–6 correspond to digests with *Bam*HI, *Bcl*I, *Eco*RI, *Eco*RV, *Hinc*II and *Rsa*I. The sizes of 1-kb DNA markers (M) are given in kilobase pairs to the left.

has almost all conserved motifs including the potential DNA-binding domain (R^{60}CDKNCVLGISINFMLKILS^{79}) that displays up to 75% similarity to the corresponding region in either human or *Saccharomyces cerevisiae*

PCNA, pol δ and RFC putative binding sites (D^{40}CSHVS^{45}), and the C-terminal region (F^{255}FLAPK^{260}) that was thought to be essential for proper folding and interaction with several binding proteins in humans (Kelman, 1997; Jónsson et al., 1998). Structure-based alignment showed that each of the strands and helices are also preserved. However, PfPCNA2 contains two unique sequence inserts (one with four amino acids located just before the βH1 region and another with seven amino acids positioned before the βI2 region) and a four-residue deletion after the βC2 motif (see Fig. 2A). Phylogenetic analysis of the eukaryotic PCNAs indicated that while vertebrates appear to have only one PCNA, the Apicomplexa protozoa and some plants may use more than one type of PCNA to carry out different types of nucleic acid metabolism such as DNA replication and repair. As shown in Fig. 2B, PfPCNA2 and TgPCNA2 fall into the same group while PfPCNA1 and TgPCNA1 group together.

3.2. Structural organisation of the Pfpcna2 gene

To determine the copy number of the *Pfpcna2* gene in the *P. falciparum* genome, 3D7A genomic DNA was digested with various restriction enzymes and analysed by Southern blotting. Hybridisation of the PC_B–PC_E DNA probe (see Fig. 1) revealed a single band in digests with *Bam*HI or *Bcl*I, for which there is no restriction site in the PC_B–PC_E fragment, and two bands in digests with *Eco*RV or *Hinc*II, for which only one restriction site exists in the probe (Fig. 3A). However, there are two bands (one predominant and the other faint) on digestion with *Eco*RI, inconsistent with the
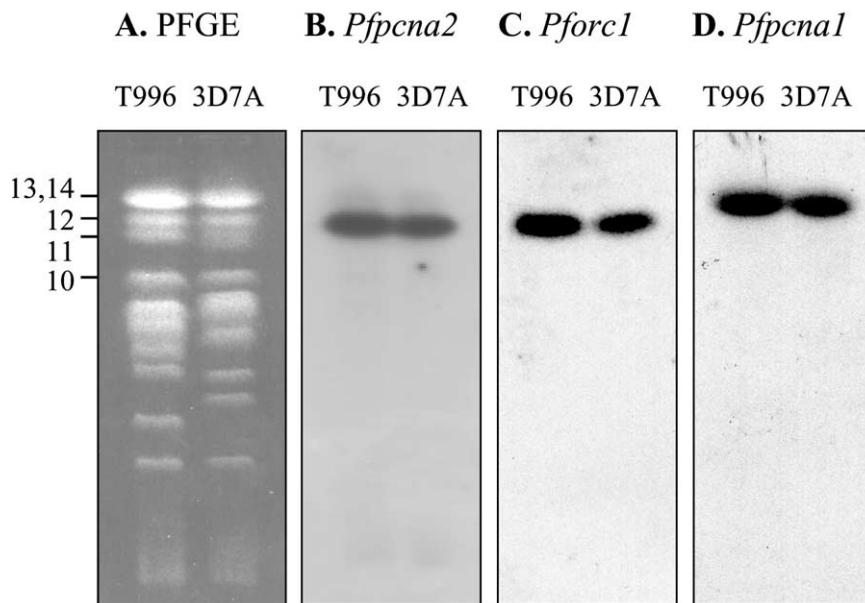
A. PFGE   **B.** *Pfpcna2*   **C.** *Pforc1*   **D.** *Pfpcna1*

Fig. 4. Chromosomal localisation of the *Pfpcna2* gene. Parasite chromosomes from *Plasmodium falciparum* 3D7A and T996 were separated by pulse-field gel electrophoresis, stained with ethidium bromide (A), blotted onto a nylon membrane and hybridised with the *Pfpcna2* gene (B), the *Pforc1* gene (C) and the *Pfpcna1* gene (D), respectively. Based on the yeast chromosome markers and hybridisation of several *P. falciparum* chromosome marker genes, the positions of chromosome 10, 11, 12, 13 and 14 were identified on the ethidium bromide-stained gel. Both *Pfpcna2* and *Pforc1* hybridised to chromosome 12, while *Pfpcna1* hybridised to chromosome 13.

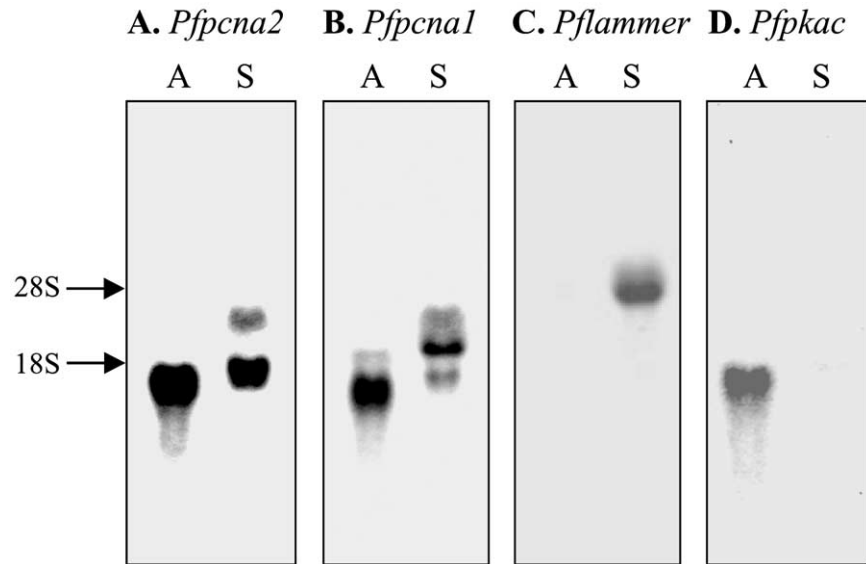**A. *Pfpcna2* B. *Pfpcna1* C. *Pflammer* D. *Pfpkac***

Fig. 5. Northern blot analysis of the *Pfpcna2* gene. Total RNA, 10 μg, extracted from the asexual erythrocytic stage (A) and the sexual erythrocytic stage (S) of *Plasmodium falciparum* (3D7A) was fractionated in a denaturing formaldehyde gel, blotted onto a nylon membrane and hybridised to radiolabeled probes. The positions of *P. falciparum* rRNA subunits (18S and 28S) are indicated by arrows. Autoradiographs of the membrane probed with the *Pfpcna2* gene (the PC_B–PC_E fragment) (A), the *Pfpcna1* gene (B), the *Pflammer* gene (C) and the *Pfpkac* gene (D). The *Pfpcna2* probe detected two transcripts of approximately 1,800 and 2,500 nucleotides: the smaller transcript exists in both asexual and sexual stages while the larger transcript occurs only in the sexual stage. *Pfpcna1* gave rise to two bands of approximately 1,600 and 2,200 nucleotides, respectively, in both asexual and sexual stages; *Pflammer* hybridised with a band of approximately 3,800 nucleotides in the sexual stage; and *Pfpkac* hybridised with a transcript of approximately 1,800 nucleotides in the asexual stage.

restriction map that does not contain any *Eco*RI-restriction site. The faint band decreased gradually with higher stringency washing conditions, indicating the presence of a PC_B–PC_E-related gene in the parasite genome. To investigate if the faint band represents the *Pfpcna1* gene (Kilbey et al., 1993), a DNA fragment corresponding to the whole coding region of *Pfpcna1* was used to probe the same blot and a different hybridisation pattern was obtained (Fig. 3B). These results suggest strongly that there are two distinguishable PCNA genes in the malaria parasite.

To investigate the chromosome location of the *Pfpcna2* gene, *P. falciparum* (3D7A and T996) chromosomes were resolved on the CHEF gel system, blotted onto a nylon membrane and hybridised with the PC_B–PC_E probe. A single band was detected corresponding to chromosome 12 (Fig. 4B). The result was repeated by hybridising another blot with the same probe (data not shown) and further confirmed by probing these blots with control probes derived from the *Pforc1* gene (Fig. 4C) and the *Pfpcna1* gene (Fig. 4D), which are known to be located on chromosomes 12 (Li and Cox, 2002) and 13 (Kilbey et al., 1993), respectively. The results further support the conclusion that two unique PCNA genes exist in *P. falciparum*.

3.3. Stage-specific expression of the Pfpcna2 gene

To obtain information on how *Pfpcna2* mRNA levels are regulated during parasite development and differentiation, a Northern blot containing equal amounts of total RNA prepared from cultures enriched in stages III–V gameto-

cytes and from mixed asexual erythrocytic stages was probed with the PC_B–PC_E cDNA fragment (see Fig. 1). Two transcripts with sizes of approximately 1,800 and 2,500 nucleotides, respectively, were detected (Fig. 5A). The smaller transcript exists in both asexual and sexual stages, migrating ahead of 18S ribosomal RNA band, whereas the larger transcript occurs only in the sexual stage. As a contol, the same blot was also hybridised with the *Pfpcna1* probe. As shown in Fig. 5B, two transcripts with sizes of 1,600 and 2,200 nucleotides were also detected by *Pfpcna1*. The 1,600 nucleotide-band is expressed predominantly in the asexual stage and weakly in the sexual stage. By contrast, the 2,200 nucleotide-transcript is expressed mainly in the sexual stage and weakly in the asexual stage. As internal controls for hybridisation to the asexual and sexual stage mRNA, the same blot was hybridised with *Pflammer*, a sexual stage-specific gene (Li et al., 2001), and *Pfpkac*, an asexual stage-specific gene (Li and Cox, 2000) (Fig. 5C and D).

3.4. Overexpression and purification of recombinant PfPCNA2

To determine whether the open reading frame of the malarial gene truly encodes the PfPCNA2 protein, a cDNA fragment corresponding to the whole coding region was subcloned into the pT7.7 and pET-11 expression vectors. PfPCNA2 was overexpressed in *E. coli* as a soluble protein and purified to near homogeneity by nickel chelate affinity chromatography. Analysis by SDS-PAGE of the
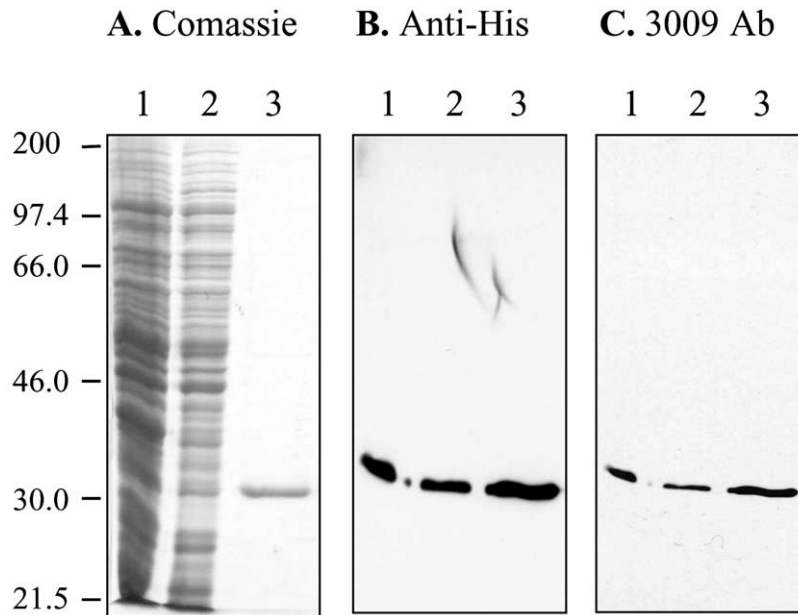
Fig. 6. Expression and purification of PfPCNA2. The recombinant protein was expressed from the pET-11-PfPCNA2 vector in *Escherichia coli* BL21-CodonPlus™(DE3)-RIL and purified with a nickel chelate affinity chromatography as described in the section of experimental procedures. After IPTG induction, samples of lysate (lane 1), supernatant (lane 2) and purified protein (lane 3) were subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue (A) or Western blotted onto a nitrocellulose membrane and probed with 1:4,000 anti-histidine monoclonal antibody (B) or with 1:2,000 anti-hPCNA 3009 polyclonal antibody (C).

homogeneous protein revealed a single band of approximately 33 kDa (Fig. 6A), consistent with the predicted Mr (31.4 kDa) of the His-tagged PfPCNA2. Human PCNA has a predicted Mr of 29.6 kDa but runs aberrantly on SDS-PAGE at 36 kDa (Kelman, 1997). The identity of the purified recombinant protein was verified by Western blotting probing with the monoclonal anti-histidine antibody (Fig. 6B) and further confirmed with the polyclonal anti-human PCNA antibody 3009 raised against the C-terminal 15 amino acid residues of human PCNA (Fig. 6C). The 3009 antibody cross-reacted only with PfPCNA2 but not with PfPCNA1 (data not shown).

### 3.5. Oligomeric properties of PfPCNA2

In order to characterise the quaternary structure of the PfPCNA2 protein, we initially carried out size-exclusion chromatography. Approximately 4 μg of the His-tagged PfPCNA2 was loaded onto a 7-ml Superdex 200 column and washed with 13 ml buffer. Fractions were collected and subjected to SDS-PAGE. After blotting, the membrane was then probed with monoclonal anti-histidine antibody. As shown in Fig. 7A, a peak with an Mr of approximately 83 kDa was obtained. The identity of PfPCNA2 in the peak was verified by Western blotting probing with the anti-histidine antibody (Fig. 7B). Human PCNA was used as a control and eluted with an Mr of approximately 92 kDa, consistent with trimers. Although on the basis of the Mr we cannot rule out completely that PfPCNA2 might form a dimer in solution, we favor the hypothesis that PfPCNA2

has a trimeric structure by homology with human and *S. cerevisiae* PCNA, as revealed by molecular modelling (data not shown). Analysis of PfPCNA2 on native gel electrophoresis gave a similar result to those from the gel filtration (data not shown). Thus, we concluded that PfPCNA2 may exist as a trimer in the parasite.

4. Discussion

PCNA plays many different roles in nucleic acid metabolism. It is an essential component of the DNA replication machinery and is required for DNA repair, recombination and transcription, as well as several other cellular processes (Cox, 1997; Kelman, 1997; Tsurimoto, 1998). Originally identified as a nuclear antigen in proliferating human cells (Miyachi et al., 1978), PCNA has been found in widely diverse organisms including *P. falciparum* (Kilbey et al., 1993). It has been thought that there is only one PCNA species in higher eukaryotic cells. In this study, we have identified a second PCNA in *P. falciparum* on the basis of several lines of evidence. Firstly, at the amino acid level PfPCNA2 only shares 29% identity and 53% similarity with PfPCNA1 (Fig. 2). In addition, Southern blot analyses revealed that the hybridisation pattern of *Pfpcna2* is completely different from that of *Pfpcna1* (Fig. 3). Moreover, chromosomal localisation studies showed that *Pfpcna2* is located on chromosome 12 while *Pfpcna1* is located on chromosome 13 (Fig. 4) (Kilbey et al., 1993). Existence of two PCNA species within the same organism is
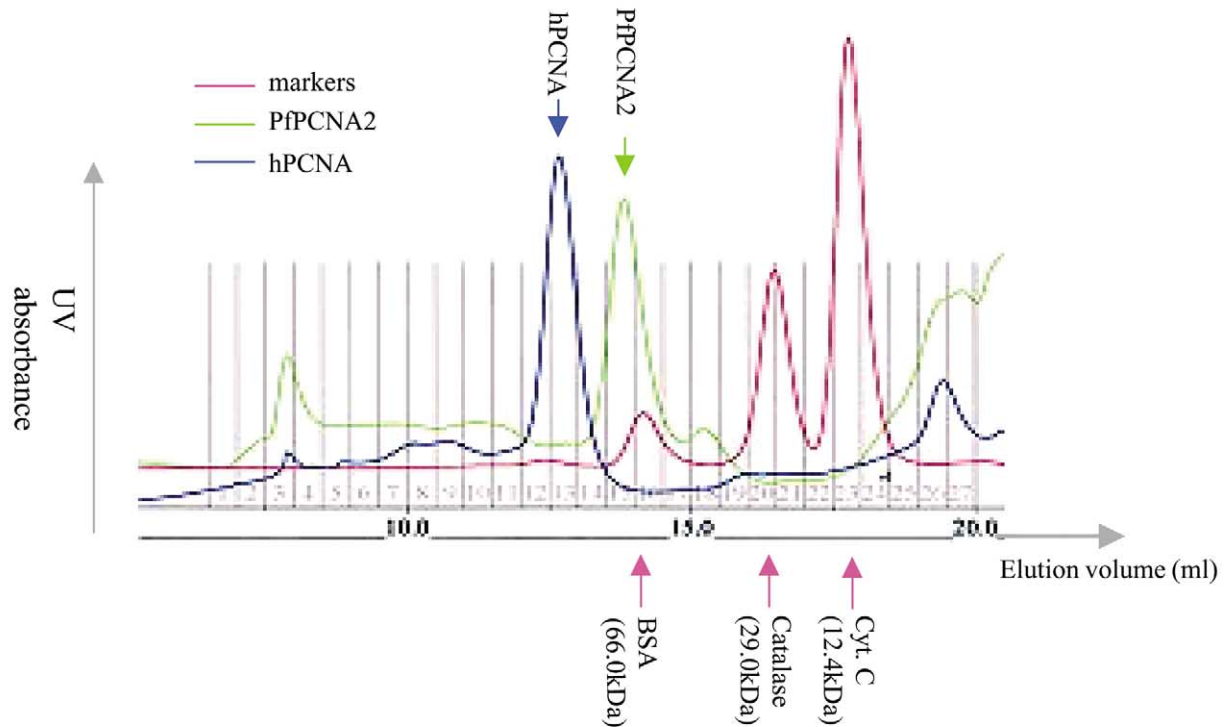
Fig. 7. Analysis of recombinant PfPCNA2 by a size-exclusion chromatography. (A). Trace shows UV absorbance at 280 nm of eluted fractions for PfPCNA2 (green line), human PCNA (hPCNA) (blue line) and protein standards (red line). (B). Fractions, 10 μl, from PfPCNA2 elution were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane and probed with 1:1,000 anti-histidine monoclonal antibody. PfPCNA2 was eluted at 83 kDa and hPCNA at 92 kDa.

quite unusual and, up to date, has occurred only in a few species (Fig. 2B) including the thermoacidophilic archaeon *Sulfolobus solfataricus* (De Felice et al., 1999), the chlorella virus *Paramecium bursaria* (Lu et al., 1995; Li et al., 1997), the Apicomplexa protozoa *Toxoplasma gondii* (Guerini et al., 2000), the carrot plant *Daucus carota* (Hata et al., 1992), the maize plant *Zea mays* (López et al., 1995, 1997) and the plant *Arabidopsis thaliana* (Lin et al., 1999; Theologis et al., 2000). Therefore, the identification of the second PCNA in *P. falciparum* may provide an opportunity that can be utilised to explore the biological consequence of two PCNA species in one eukaryotic organism.

Analysis of the amino acid sequences revealed that PfPCNA2 consists of two similar domains and contains almost all motifs conserved in the PCNA family, suggesting that PfPCNA2 may possess essential functions like the human homologue (Kelman, 1997). However, compared with human and *S. cerevisiae* PCNA, PfPCNA2 contains two unique amino acid sequence inserts (one located just

before the βH1 region and another positioned before the β12 region) and a sequence deletion after the βC2 motif (see Fig. 2A), suggestive of a different subset of functions in PfPCNA2. It is worth noting that PfPCNA1 also possesses an amino acid insert with nine residues at the same position as in PfPCNA2, near the C-terminus (see Fig. 2A). The C-terminal region of PCNA has been reported to be essential for interaction with its binding proteins such as DNA pol ε, RFC, Fen1, GADD45, p21 and DNA methyltransferase (Tsurimoto, 1998; Kelman and Hurwitz, 1998). Therefore, the amino acid insert near the C-terminus (before βI2) may affect the interaction of PfPCNA2 with some of the binding proteins.

PCNA is functionally analogous to the β-subunit of *E. coli* DNA polymerase III (pol III) and the T4 bacteriophage gene 45 protein which function as DNA sliding clamps (Kelman, 1997). Both human and yeast PCNA have been shown to form homotrimers in solution (Bauer and Burgers, 1990; Yao et al., 1996), and crystallised as a trimeric ring

with a six-fold axis of symmetry derived from two globular domains per monomer (Krishna et al., 1994; Gulbis et al., 1996). However, the pol III β-subunit exists as a dimeric ring, each consisting of three repeating units in six-fold symmetry (Kong et al., 1992). It is therefore quite possible that eukaryotic PCNA may also exist as a homodimer. It has been known that two distinct PCNA genes are expressed in carrot (Hata et al., 1992), one encoding a typical size PCNA (264 amino acids) that might form a trimer and the other coding for a longer form (365 amino acids) that might form a dimer (Kelman, 1997). In addition, apart from the 36 kDa protein, a 43 kDa peptide can also be recognised by an anti-PCNA antibody during *Xenopus laevis* oogenesis (Leibovici et al., 1990). By using both size-exclusion chromatography and native gel electrophoresis, we have shown that recombinant PfPCNA2 protein seems to form a trimer in solution (Fig. 7). Molecular modelling studies based on the structures of human and yeast PCNA (Krishna et al., 1994; Gulbis et al., 1996) suggest that PfPCNA2 may form a trimeric ring. Therefore, we propose that PfPCNA2 may serve as a sliding platform which is capable of interaction with a wide array of proteins and stabilises associations of these proteins with DNA template, and consequently regulates their activities.

Northern blot analyses revealed two transcripts with sizes of 1,800 and 2,500 nucleotides, respectively. The former is expressed in both asexual and sexual stages, whereas the latter is specifically expressed in the sexual stage, suggesting that PfPfPCNA2 may play an essential role in DNA metabolism at different stages of the parasite. It is worth noting that the *Pfpcna1* gene also produced two transcripts with sizes of 1,600 and 2,200 nucleotides, respectively (Kilbey et al., 1993), which were probably accumulated in the trophozoite stage (Horrocks et al., 1996). Accordingly, to exclude the possibility that *Pfpcna2* may cross-react with *Pfpcna1* in Northern blotting, the same blot was hybridised with the *Pfpcna1* probe. Interestingly, the smaller transcript is expressed predominantly in the asexual stage and weakly in the sexual stage; in contrast, the larger transcript is mainly present in the sexual stage and weakly in the asexual stage (see Fig. 5), implying that PfPCNA1 may have broader functions than originally thought (Kilbey et al., 1993; Horrocks et al., 1996), that is, it functions not only in the asexual stage but also in the sexual stage. The quantity of DNA in mature gametocytes of *P. falciparum* has been shown to reach the diploid value (Janse et al., 1988), implying either complete genome duplication or selective gene amplification per haploid genome. Upon activation, the microgametocyte develops rapidly in the mosquito midgut (gametogenesis). Three successive rounds of genome replication are completed within 10 min, raising the DNA contents to octoploid values just before exflagellation, indicating that the genome duplication rate of the malaria parasite is extremely high, probably among the highest recorded. Assuming that the rate of replication fork movement in *Plasmodium* is similar to that in other eukaryotes, at least 1,300 origins of replication would be needed to achieve this rate of replication (Janse et al., 1986). The high level expression of PfPCNAs in both asexual and sexual stages of *P. falciparum*, therefore, is consistent with their potential role in DNA replication during the erythrocytic cycle and the sexual stage development, particularly during gametogenesis. However, the fact that both PCNA species are expressed in both asexual and sexual stages gives rise to more curious questions. The two molecular species may play the same role in DNA replication of the parasite but have different functions at different stages of the parasite in DNA repair, recombination and transcription, as well as other cellular processes such as cell cycle control and maintenance of chromosome structures (Kelman, 1997; Shibahara and Stillman, 1999). Identification of novel PfPCNA-interacting proteins will help afford new insights on biological functions of these two PCNA species in the human malarial pathogen.

Acknowledgements

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