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Short communication

A putative protein serine/threonine phosphatase from *Plasmodium falciparum* contains a large N-terminal extension and five unique inserts in the catalytic domain[1]

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Plasmodium falciparum possesses a complex life cycle involving two different hosts and interactions with multiple cell types. During the various stages of the life cycle the parasite undergoes cell growth and division, development and differentiation. Sexual stage development, gametocytogenesis in the host and gametogenesis in the mosquito, is accompanied by biochemical and morphological changes in the parasite. The molecular and cellular mechanisms involved in regulation of proliferation, development and differentiation of *P. falciparum* are unclear. However, inhibitors of protein kinases and phosphatases can interfere with parasite growth [1], suggesting a requirement for phosphorylation–dephosphorylation in control of the parasite life cycle. Reversible phosphorylation has been shown to play an important role in invasion of erythrocytes by merozoites [2] and in intraerythrocytic growth and development [3]. Recently, a number of protein serine/threonine kinase genes have been isolated from *P. falciparum* [4–7].

Abbreviations: PPP, PP-1/PP-2A/PP-2B protein serine/threonine phosphatase family; PfPP-α, *Plasmodium falciparum* protein serine/threonine phosphatase α; PfPP-β, *Plasmodium falciparum* protein serine/threonine phosphatase β; PFGE, pulse-field gel electrophoresis.

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[1] *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDJB databases under the accession number U88869.

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To understand the cell cycle events and signal transduction pathways regulated by reversible phosphorylation in *P. falciparum*, apart from the protein kinases, the relevant protein phosphatases involved have to be identified and characterized. In a previous paper, we have described a PP-2A-like protein serine/threonine phosphatase from *P. falciparum* [8]. Here, we report the molecular characterization of another novel PP-1/PP-2A/PP-2B (PPP)-related protein serine/threonine phosphatase from *P. falciparum*. This phosphatase, termed *P. falciparum* protein serine/threonine phosphatase α (PfPP-α), not only contains five unusual inserts in the catalytic domain but also has the largest N-terminal extension amongst known protein phosphatases of the PPP family.

On the basis of two highly conserved motifs (GDYVDRG and DLLWSDP) in the catalytic domain of the PPP family [9], two degenerate oligonucleotide primers, termed P1 (5′ GGIGAT-TAT/CGTIGAT/CA/CGIGG 3′, nucleotides 2043–2062) and P3 (5′ GGATCIIICCAIAA/GIAA/GA/GTC 3′, nucleotides 2415–2434), were constructed and then used in PCR to amplify the desired gene from 3D7A genomic DNA (data not shown). The sequence of one PCR clone (P1–P3) revealed a 392 bp insert encoding 131 amino acids of a protein serine/threonine phosphatase-like protein. The A + T content and codon usage of the nucleotide sequence are typical of the coding region of *P. falciparum* genes. Southern blotting confirmed that the P1–P3 clone is derived from parasite DNA (data not shown). Therefore, the P1–P3 clone was employed to screen a λGEM-12 genomic library [6]. Restriction digestion of one positive plaque (λP1) DNA with a variety of enzymes gave rise to a fragment of $\approx$1.5 kb, which was shown by hybridization to contain the target gene. The fragment was cloned into the pUC19 vector (clone P2-2). Sequence data showed that the P2-2 clone contained only a partial sequence of the desired gene. Accordingly, attempts were made to clone other fragments including a 3.0 kb *Bam*HI fragment and a 4.5 kb *Eco*RI fragment. This proved unsuccessful. Consequently, vectorette libraries were constructed and screened by PCR. Based on the southern blotting data and the sequence obtained from the P1–P3 PCR and P2-2 pUC19 clones, a partial restriction map of the gene was established (Fig. 1A). Two specific primers, PP1 (to obtain further sequence in the 3′ direction) and PP10 (to obtain further sequence in the 5′ direction), were used in PCR to walk outwards in both directions. The sequence data thus acquired permitted construction of the PP14 primer and subsequent complete sequencing across the gene (Fig. 1A).

The sequence derived from the overlapping genomic clone and vectorette PCR fragments consists of 3128 bp and contains one open reading frame with a putative ATG start codon at nucleotide 174 and a TAA stop codon at nucleotide 2799. The ORF encodes a protein of 875 amino acids with a predicted molecular mass of $\approx$99 kDa. The predicted PfPP-α protein comprises two distinct domains. The N-terminal segment consists of 516 amino acids (see below), whereas the C-terminal segment has 359 amino acids and contains a region of high similarity to the catalytic subunits of the other protein phosphatases in the PPP family. The catalytic domain of PfPP-α contains the majority (40 out of 45) of the conserved residues in the PPP family [10]. Some of these residues have been known to be involved in metal cation binding, substrate recognition, catalysis and toxin binding by genetic mutagenesis [11] and more recently by crystallographic studies of rabbit and human PP-1 [12]. These include (relative to the residue numbers of rabbit muscle PP-1α) [12]: (a) Asp95, Arg96, Asn124, His125, Asp208 and Tyr272, forming part of the substrate binding and catalytic sites; (b) Asp64, His66, Asp92, Asn124, His173, and His248, which coordinate Mn^{2+} and Fe^{2+} binding in PP-1; (c) Arg246, being essential for the function of phosphatase enzymes; (d) Tyr272, involved in binding to toxins such as tautomycin, calyculin A and okadaic acid; and (e) the motif Ile121-Arg122-Gly123-Asp124-His125-Glu126, which is a signature of the catalytic domain of serine/threonine specific protein phosphatases in the PPP family. However, five well-conserved residues (Gly80, Pro196, Gly199, Arg211, and Pro270) in the PPP family [10] are replaced in PfPP-α by Lys80, Ser196, Pro199, Thr211, and Thr270, respectively. Database searches revealed further that the catalytic domain

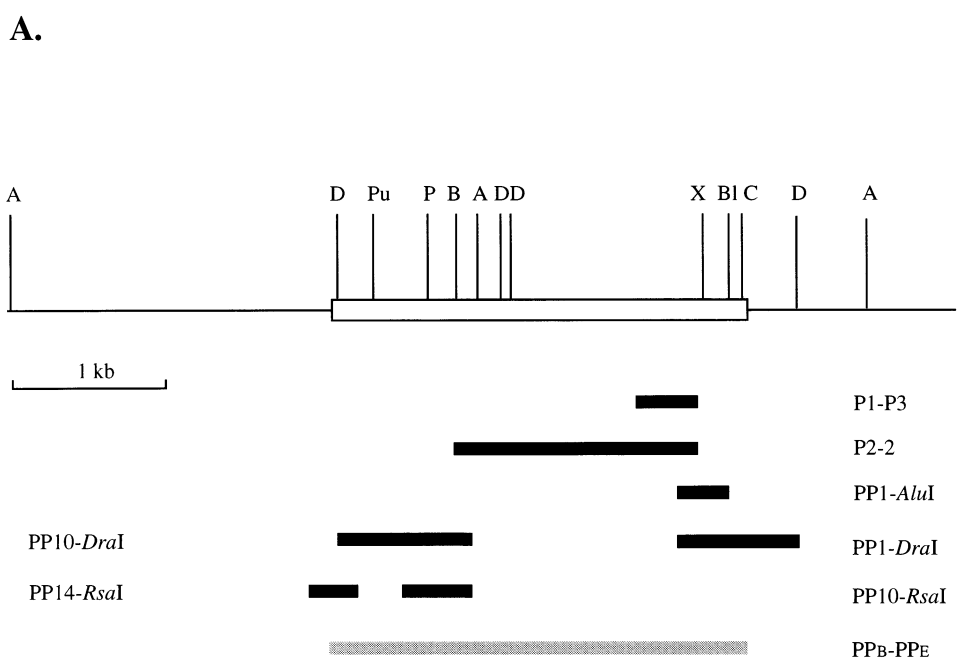

Fig. 1. (A) A schematic representation of a partial restriction map of the *Pfpp-α* gene and the overlapping clones of the genomic library and PCR fragments used to determine the nucleotide sequence of *Pfpp-α*. A, *Acc*I; B, *Bam*HI; Bl, *Bgl*II; C, *Cla*I; D, *Dra*I; P, *Pst*I; Pu, *Pvu*II, and X, *Xba*I. The open box indicates the coding region of the *Pfpp-α* gene. The PPB-PPE fragment is derived from genomic DNA. (B) Alignment of the predicted amino acid sequences of PfPP-α with human PP-1α and PP-2Aα. The GenBank™/EMBL database accession numbers are as follows: *P. falciparum* PfPP-α, U88869; human HuPP-1α, X70848; human HuPP-2Aα, X12646. The sequences were aligned with the CLUSTAL W (1.60) multiple sequence alignment programme. Identical amino acids are indicated by an asterisk and conservative changes by a dot. Inserts I-V are labelled on the top of sequences. The positions of secondary structural elements [12] are shown above the sequences. (C) A schematic representation of the structural comparison of PfPP-α with protein phosphatases in the PPP family. The black boxes denote the conserved catalytic domain of protein phosphatases in the PPP family and the open boxes represent the N- or C-terminal extension of protein phosphatases. The amino acid numbers of each phosphatase are shown to the right.

of PfPP-α is more closely related to PP-1 than to PP-2A (Fig. 1B). It shares 48–54% similarity and 40–46% identity with PP-1 from the different species, in comparison to the 42–46% similarity and 33–38% identity with PP-2A and to the 36–42% similarity and 31–35% identity with PP-2B of other organisms. Sequence comparison of PfPP-α with PP-1 showed that PfPP-α contains a number of residues conserved in the PP-1 subfamily (see Fig. 1B). However, it is different from PP-1 in several respects. There are changes in more than 40 residues which are well conserved in all members of the PP-1 group [10], including some within the Gly274-Glu275-Phe276-Asp277 motif in the β12-β13 loop of PP-1, which is thought to be involved in the binding of okadaic acid [11]. In addition, PfPP-α contains five unique inserts in the catalytic domain: insert I, consisting of eight amino acids, located within the αA helix based on the tertiary structure of PP-1 [12]; insert II, containing 14 amino acid residues, upstream of the β3 strand; insert III, comprising six amino acids, between the αE and αF helices; insert IV, composed of six amino acid residues, positioned upstream of the αH helix; and insert V, possessing eight amino acids, following the β8 strand. To investigate the potential function of these inserts, the three-dimensional structure of the catalytic domain of PfPP-α was modelled using the computer programme Swiss-Pdb Viewer (Version 2.11) on the basis of the crystal structure of the catalytic domain of rabbit muscle PP-1α [12] (data

B.

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                                                                    αA'           β1'
PfPP-α   {480}AFLVRKISIDKLEEEGRKIN  NGVLCTPVNYISEFKNTVYD  KIITTLLNPNITQFEIQYNH  540
HuPP-1α       --------------------  ----------MSDSEKLNLD  SIIGRLLEVQGSRPGKNVQL   30
HuPP-2Aα      --------------------  -----------------MDE  KVFTKELDQWIEQLNECKQL   23
                                                       .   .    *      .    .

               Insert I     αA                   β1     β2            αB
PfPP-α        NSESIFIIPWANISVLCSIV  IDIFKQEDMVLKLRAPIKIY  GDIHGQYYDLMRMFQLYKCP  600
HuPP-1α       TE--------NEIRGLCLKS  REIFLSQPILLELEAPLKIC  GDIHGQYYDLLRLFEYGGF-   81
HuPP-2Aα      SE--------SQVKSLCEKA  KEILTKESNVQEVRCPVTVC  GDVHGQFHDLMELFRIGGK-   74
                        .  **       .*    .  .  . .*. .   **.***. **. .*

               Insert II       β3                 αC            β4
PfPP-α        VEEDLGEKLNAIGDIDSNDY  LFLGDYVDRGSNSLEVICLL  FALKCKYPKQIHLIRGNHED  660
HuPP-1α       -------------PPESN-Y  LFLGDYVDRGKQSLETICLL  LAYKIKYPENFFLLRGNHEC  127
HuPP-2Aα      -------------SPDTN-Y  LFMGDYVDRGYYSVETVTLL  VALKVRYRERITILRGNHES  120
                            ..* *   **.*******  *.* ..**    * * .*      ..*****

               αD      αE      Insert III   αF              β5     β6
PfPP-α        VAINSLYGFQEECKRRLKED  VTDKDSCWYQINQVFEWLPI  GAIVEDKILCVHGGIGKSIN  720
HuPP-1α       ASINRIYGFYDECKRRY---  ---NIKLWKTFTDCFNCLPI  AAIVDEKIFCCHGGLSPDLQ  181
HuPP-2Aα      RQITQVYGFYDECLRKYG--  ---NANVWKYFTDLFDYLPL  TALVDGQIFCLHGGLSPSID  175
                *. .*** .** *.              *    .  *  **.   *.*. .* * ***.    .

               αG         Insert IV       αH    β7           β8   Insert V    β9
PfPP-α        QISDISQLKRPLVVSQVPQN  LNEQKVTDLLWSDPTDNDSI  LGTIPNDIRDPDGTGHIVKY  780
HuPP-1α       SMEQIRRIMRPTDVP-----  -DQGLLCDLLWSDPDKDVQG  WGEND--------RGVSFTF  227
HuPP-2Aα      TLDHIRALDRLQEVP-----  -HEGPMCDLLWSDPD-DRGG  WGISP--------RGAGYTF  220
               .  *  . *   *           .  ..*******         *            *    .

               αI            β10          β11     β12                 β13
PfPP-α        GPDRVHKFLEENDLQLIIRA  HECVMDGFERFAGGKLITLF  SATNYCNSHKNAGALLFIRR  840
HuPP-1α       GAEVVAKFLHKHDLDLICRA  HQVVEDGYEFFAKRQLVTLF  SAPNYCGEFDNAGAMMSVDE  287
HuPP-2Aα      GQDISETFNHANGLTLVSRA  HQLVMEGYNWCHDRNVVTIF  SAPNYCYRCGNQAAIMELDD  280
              * .    *    . * *. **  *. * .*.         ..*.*  ** ***     *  *.. .

               β14
PfPP-α        DLTVIPKLIYPAKD------  --EGDSLIHGKKYEEKCPYI  DMY                   875
HuPP-1α       TLMCSFQILKPADKNKGKYG  QFSGLNPGGRPITPPRNSAK  AKK                   330
HuPP-2Aα      TLKYSFLQFDPAPR------  R--GE-----PHVTRRTPDY  FL-                   309
               *         **           *              .
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Fig. 1. (*Continued*)

not shown). Although the three-dimensional structure of the inserts is not known, secondary structure algorithms predict a loop conformation for the residues within these inserts. The positions of the inserts are clearly separate from the catalytic region and therefore probably do not play a direct role in catalysis. They appear to be associated with the periphery of the catalytic domain and may therefore interact with regulatory components that are yet to be defined. These features may place PfPP-α in a unique category within the PPP family.

C.

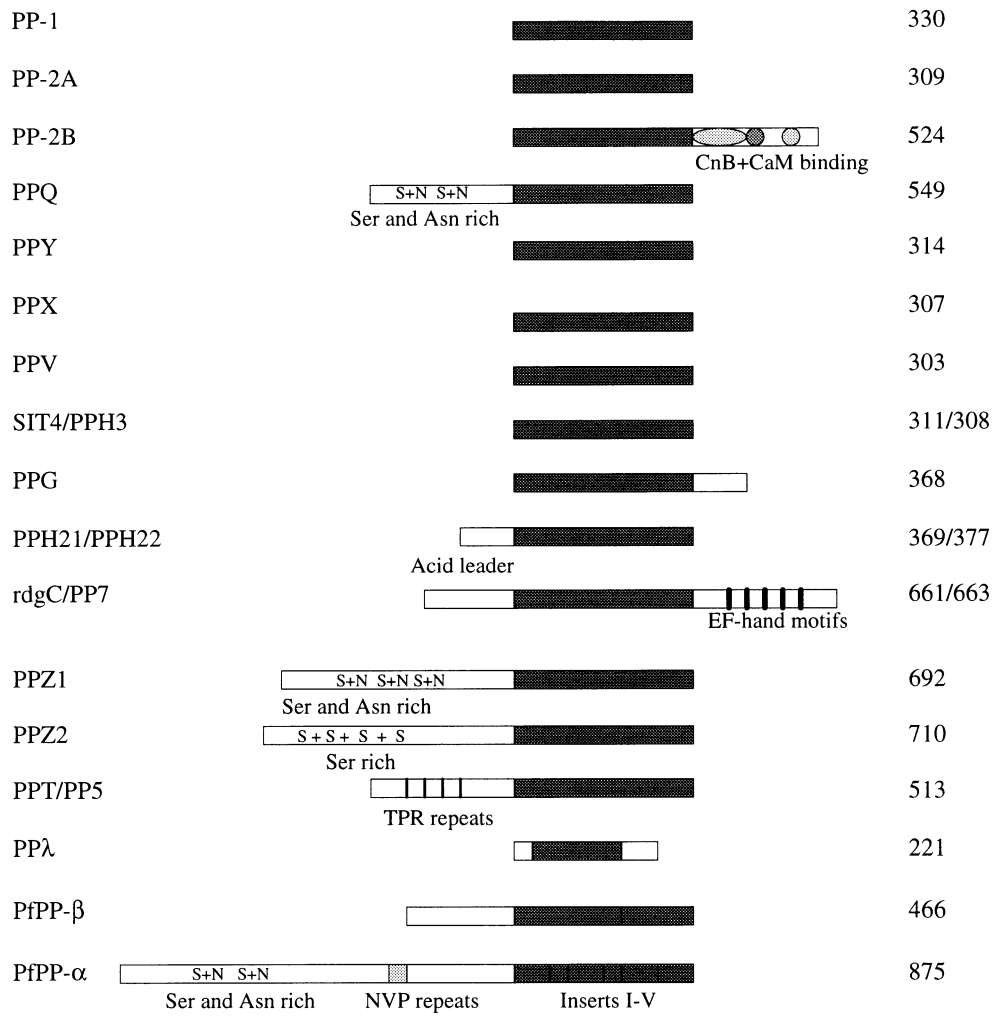

Fig. 1. (*Continued*)

The amino-terminal region of PfPP-α is not related to any previously described protein serine/threonine phosphatases and has several interesting features. Firstly, there are six copies of a degenerate trimer N(I)VP(Q) repeat at residue positons 419–436, which is reminiscent of the NVDP repeats found in the *P. falciparum* circumsporozoite protein. Secondly, in contrast to the catalytic domain, this region is relatively rich in serine (8.5%), asparagine (9.5%) and threonine (6.2%). The N-terminal segment represents about 59.0% of PfPP-α in size, however, 71.0% of serine (44/62) and asparagine (49/69), and 72.7% of threonine (32/44) residues are located within the N-terminal portion. It is noteworthy that the N-terminal domains of PPQ (comprising 236 residues), PPZ1 (containing 360 amino acids) and PPZ2 (consisting of 395 residues) are also rich in serine and/or

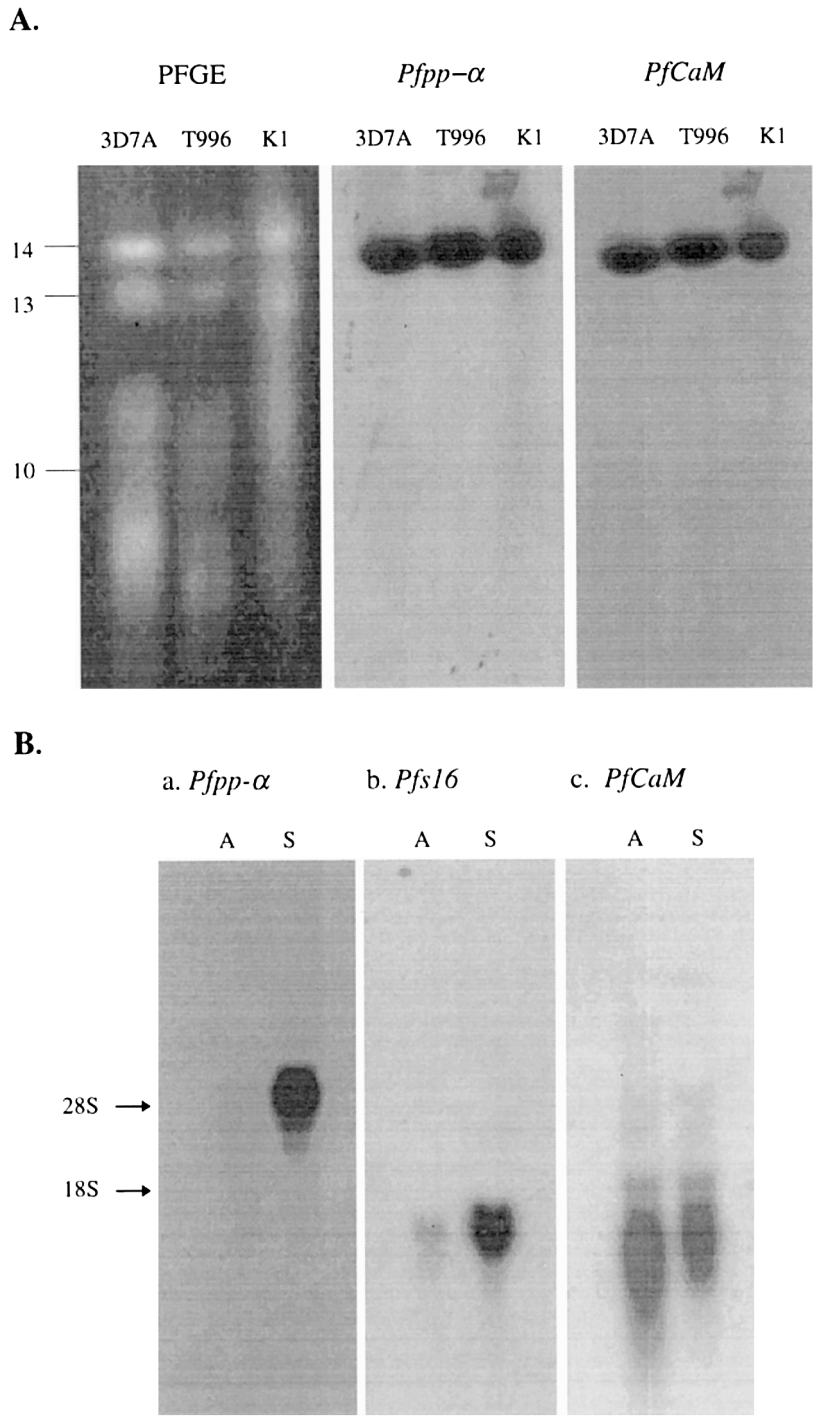
A.
PFGE
Pfpp−α
PfCaM
3D7A T996 K1
3D7A T996 K1
3D7A T996 K1
14
13
10
B.
a. Pfpp-α
b. Pfs16
c. PfCaM
A S
A S
A S
28S
18S

Fig. 2.

asparagine [13–15]. Thirdly, the N-terminal domain contains multiple potential phosphorylation sites for a range of known protein kinases [16]. For instance, if the sequence R/K-X-X-S/T is considered to be the minimal recognition motif for cAMP-dependent protein kinase, more than ten serine/threonine residues may act as potential phosphorylation sites. Similarly, if the motif S/T-X-K/R is a minimal requirement for protein kinase C-dependent phosphorylation, at least eight serine/threonine residues could be considered as potential phosphorylation sites. Thus, there is a strong possibility of regulation of PfPP-α by reversible phosphorylation. It is well known that protein serine/threonine phosphatase activity can also be regulated through the interaction of the catalytic subunits with regulatory proteins [17]. Therefore, it is also tempting to speculate that the N-terminal extension of PfPP-α may regulate the specificity of the C-terminal catalytic domain by binding one or more accessory subunits that target the enzyme to its correct location. Finally, the N-terminal segment is the largest extension amongst the known protein phosphatases in the PPP family yet described. The phosphatases carrying an N-terminal extension include *Drosophila* rdgC/human PP7 [18,19], *Saccharomyces cerevisiae* PPH21, PPH22 [20], CNA1/CMP1 [21], CNA2/CMP2 [22], PPQ [13], PPZ1 [14], PPZ2 [15], PPT (PP5 in human) [23] and *P. falciparum* PfPP-β [8]. PPZ2 possesses the largest N-terminal extension ($\approx$395 residues) amongst the above mentioned phosphatases. However, the N-terminal region (516 amino acids) of PfPP-α is substantially longer than that of PPZ2.

In order to reveal the precise relationship of PfPP-α with other protein phosphatases identified so far, comparison of the overall sequence of PfPP-α with other relevant phosphatases in the PPP family was carried out (Fig. 1C). The results clearly show that PfPP-α is a member of the PPP family but cannot be assigned to any particular subgroup with the PPP family. Therefore, PfPP-α seems to comprise a distinct subgroup in the PPP family.

To determine the copy number of the *Pfpp-α* gene in the parasite genome, southern blot analyses were performed using genomic DNA (3D7A) digested with a number of restriction enzymes. Hybridization of the blots were performed with the P1–P3 probe and the P2-2 fragment, respectively. The results (data not shown) are consistent with the restriction map (see Fig. 1A), indicating strongly that *Pfpp-α* is a single-copy gene per haploid genome. To determine the chromosomal location of the *Pfpp-α* gene, hybridization of the P2-2 clone was carried out using a pulse-field gel electrophoresis (PFGE) blot containing 3D7A, T996 and K1 chromosomes. A single band was detected corresponding to chromosome 14 (Fig. 2A). This was repeated using the same probe with an independent PFGE blot (data not shown). The result was further confirmed by re-probing the same blots with the calmodulin gene (*PfCaM*) which is located on chromosome 14. The single band result on the PFGE blots also supports the conclusion that *Pfpp-α* is a single-copy gene in the parasite genome.

Analysis of *Pfpp-α* mRNA expression in the erythrocytic life cycle stages of *P. falciparum* may give some clues as to its possible function in the

Fig. 2. (A). Chromosome localization of *Pfpp-α*. Parasite chromosomes from *P. falciparum* 3D7A, T996 and K1 were separated by pulse-field gel electrophoresis, stained with ethidium bromide, blotted onto a nylon membrane and hybridized with radiolabeled probes. According to the yeast chromosome markers and hybridization of several *P. falciparum* chromosome marker genes, the positions of chromosome 10, 13, and 14 were identified on the ethidium bromide-stained gel. *Pfpp-α* and *PfCaM* both hybridized to chromosome 14. (B) Northern blot analysis of the *Pfpp-α* gene. Ten μg of total RNA extracted from the asexual erythrocytic stages (A) and sexual erythrocytic stages (mainly stage III–V) (S) of *P. falciparum* (clone 3D7A) were fractionated in a denaturing formaldehyde gel, blotted onto a nylon membrane and hybridized to radiolabeled probes. The positions of *P. falciparum* rRNA subunits (18S and 28S) are indicated by arrows. (a), (b) and (c) are autoradiographs of the membrane probed with the *Pfpp-α* gene, the *Pfs16* sexual stage-specific gene and the calmodulin housekeeping gene (*PfCaM*), and exposed for 24 h, 50 min and 96 h, respectively. *Pfpp-α* detected a transcript of $\approx$3900 nucleotides in the sexual erythrocytic stages, *Pfs16* hybridized with a band of $\approx$1400 nucleotides in the sexual erythrocytic stages and *PfCaM* hybridized with two bands of 1300, and 1000 nucleotides from the parasite.

parasite development and differentiation. Northern blot analysis was performed by using the P2-2 probe. A transcript of ≈ 3900 nucleotides in size was only detected in the lane containing sexual stage RNA, migrating just behind the 28S ribosomal RNA band (Fig. 2B). This suggests that PfPP-α is involved in sexual stage-specific events. It is known that in vertebrates protein dephosphorylation plays a key role in a number of testicular functions including germ cell meiosis [24] and sperm motility [25]. Testis-specific forms of PP-1 and PP-2B have been identified in mammalian systems [25]. Recently, a male specific protein phosphatase, PPY, has been localized in somatic cells of the *Drosophila* testis [26]. In the plant Arabidopsis, it has been also shown that a PP-1 homologue is predominantly expressed in the tapetum, the developing and mature pollen and in the ovaries [27]. Interestingly, PfKIN, an SNF1-type protein kinase, and PfMRP, a MAP kinase-related protein, have been reported to be specifically expressed in the sexual stages of the life cycle [5,7]. These findings imply that reversible protein phosphorylation events may be particularly important in regulation of sexual stage development in *P. falciparum*. Various inhibitors and activators of signal transduction pathways have been shown to influence events such as gametocytogensis and exflagellation [28,29]. DNA replication, cell division (exflagellation), and fertilization occur sequentially in the sexual stage. Interestingly, Pfcrk-1 and Pfmrk, two *P. falciparum* Cdk-related protein kinases, have been recently shown to be expressed predominantly in gametocytes [4,6]. Taken together it is concluded that PfPP-α may play an important role in the control of cell cycle and signal transduction involved in the development of sexual stage and/or in fertilization.

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