

Research

Characterisation and expression of a PPI serine/threonine protein phosphatase (PfPPI) from the malaria parasite, *Plasmodium falciparum*: demonstration of its essential role using RNA interference

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Abstract

Background: Reversible protein phosphorylation is relatively unexplored in the intracellular protozoa of the *Apicomplexa* family that includes the genus *Plasmodium*, to which belong the causative agents of malaria. Members of the PPI family represent the most highly conserved protein phosphatase sequences in phylogeny and play essential regulatory roles in various cellular pathways. Previous evidence suggested a PPI-like activity in *Plasmodium falciparum*, not yet identified at the molecular level.

Results: We have identified a PPI catalytic subunit from *P. falciparum* and named it PfPPI. The predicted primary structure of the 304-amino acid long protein was highly similar to PPI sequences of other species, and showed conservation of all the signature motifs. The purified recombinant protein exhibited potent phosphatase activity *in vitro*. Its sensitivity to specific phosphatase inhibitors was characteristic of the PPI class. The authenticity of the PfPPI cDNA was further confirmed by mutational analysis of strategic amino acid residues important in catalysis. The protein was expressed in all erythrocytic stages of the parasite. Abrogation of PPI expression by synthetic short interfering RNA (siRNA) led to inhibition of parasite DNA synthesis.

Conclusions: The high sequence similarity of PfPPI with other PPI members suggests conservation of function. Phenotypic gene knockdown studies using siRNA confirmed its essential role in the parasite. Detailed studies of PfPPI and its regulation may unravel the role of reversible protein phosphorylation in the signalling pathways of the parasite, including glucose metabolism and parasitic cell division. The use of siRNA could be an important tool in the functional analysis of *Apicomplexan* genes.

Background

Reversible protein phosphorylation is gaining recognition as a potentially important mechanism of post-translational regulation in protozoan parasites, especially those belonging to the *Apicomplexan* family. The dephosphorylation of phosphoproteins is universally catalyzed by protein phosphatases that are classified into two major functional groups, protein tyrosine phosphatase (PTP) and protein serine/threonine phosphatase (PP) although enzymes with various degrees of dual-specificity are also encountered [1–5]. The majority of Ser/Thr phosphatases belong to three classical groups, namely PP1, PP2A, and PP2B (calcineurin), and possess similar primary structures in their catalytic cores [2,3,6]. PP1, in particular, exhibits an extremely high degree of sequence conservation through evolution, and its orthologs and isoforms are found in all eukaryotic cells [6,7]. In various organisms, PP1 regulates such diverse cellular processes as cell cycle progression, protein synthesis, carbohydrate metabolism, transcription, and neuronal signaling [3,7], underscoring its profound importance in biology. The PP1 and PP2A phosphatases are differentially affected by natural toxins such as okadaic acid (OA) and microcystin-LR. For example, the characteristic IC₅₀ values for OA fall in the range: PP2A, 1–5 nM, PP1, 20–80 nM, whereas PP2B is highly resistant to both [2,3,7]. In contrast, tautomycin affects PP1 and PP2A nearly equally, but fails to inhibit other phosphatases [8].

In the past few years, a number of phosphatase activities and putative sequences have been reported in *P. falciparum* [9]. These include a PP2A [10], a PP2B-like activity [10], a unique chimeric PP2C [11], two putative sexual-stage phosphatases – PP α [12] and PP β [13], and a tetratricopeptide repeat-containing phosphatase, PP5 [14]. Preliminary studies revealed the presence of a protein phosphatase activity in crude extracts of RBC-grown *P. falciparum* that exhibited toxin-sensitivity resembling that of PP1 [15]. Uninfected RBC, in contrast, possessed mainly a PP2A-like activity. Because of its potential importance in a variety of signalling pathways of the parasite, we have turned our attention to defining the PP1 phosphatase and its regulation in *P. falciparum*.

In this communication, we report the exact sequence of a PP1 cDNA in *P. falciparum*, the corresponding gene sequence in *P. falciparum* chromosome 14, the enzymatic properties of the recombinant enzyme, and its inhibition by mammalian physiological PP1-inhibitors, namely, inhibitor-1 (I-1) and inhibitor-2 (I-2). Post-transcriptional gene silencing using synthetic short interfering RNA (siRNA) molecules has been recently used to ablate specific mRNAs and thus, produce phenotypic mutations in specific genes [16,17]. We have adopted this technology to knockdown specific gene products in RNA viruses that are

obligatory intracellular parasites [18]. In the present study, we have successfully used a similar strategy to generate phenotypic PP1-deficient *P. falciparum* parasites.

Results and Discussion

Identification of the PfPP1 cDNA sequence

Various pairs of oligodeoxynucleotide primers were designed on the basis of the PlasmoDB-predicted mRNA sequence (Gene chr14_1.phat_133), and employed in reverse transcription-PCR (RT-PCR) amplification using Pf3D7 total mRNA as template. Based on the prediction, primers 5' ATGGCATTAGAAATAGATATAGATAATG 3' (primer A in Fig. 1, the start codon in bold) and 5' TTATTCCGACAAAAAGAAATATATGG 3' were first tested, but no product was obtained. Since there was no other ATG within a reasonable distance upstream that was in the same reading frame, we proceeded on the assumption that the 3'-end of the mRNA might be different. Thus, the second primer was replaced by a series of nested primers (based on the genomic sequence), each of which was paired with primer A in RT-PCR. The combination of primer A and the primer 5' TTTTAAATTTGCTGCTTCTTTTTTCC 3' (Fig. 1) eventually produced a RT-PCR product that was cloned into pGEM-T vector and sequenced. The cDNA sequence contained a 915-nucleotide long open reading frame corresponding to a polypeptide 304 amino acid in length and ending with a TAA stop codon.

Comparison of the cDNA sequence with the genomic sequence (in Chromosome 14 at TIGR) revealed that the coding sequence is divided into five exons, of which the first two are the largest and contain most of the catalytic core of the phosphatase (Fig. 1 and 2). The intron sequences are pronouncedly more AT-rich than exons, and contained homopolymeric repeats, a feature which, in our experience, is common in *Plasmodium* genes.

BLAST analysis of the predicted primary structure of the protein revealed its clear identity with the PP1 class (Fig. 2). It is to be mentioned that among all the Ser/Thr phosphatases, PP1 has been subjected to the most extensive structure-function analysis [19–26]. In fact, it was one of the first phosphatases for which the three-dimensional structure was solved [26,27]. A representative alignment in Fig. 2 demonstrates the high sequence conservation between the human and *Plasmodium* PP1 sequences. The catalytic core of all members of the PP1 and PP2 families are very conserved, and roughly corresponds to residues 5–260 of PfPP1 (Fig. 2). This region contains all the signature motifs and conserved residues that have been shown to be important for the fundamental steps of catalysis, including substrate binding, metal ion coordination, and interaction with the phosphate group [19,26–28]. It is to be noted that at 304 amino acid residues, PfPP1 is the

Primer A

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ATGGCATTAGAAATAGATATAGATAAATGTAAATATCAAACACTAATAGAAGTTCGTGGAACTAGACCAGGAAAAAATGTTAATTT
M A L E I D I D N V I S K L I E V R G T R P G K N V N L
GACAGAAAATGAAATAAAAATATTATGTTTATCAAGTAGGGAAAATATTTTAAACCAACCAATTTTATTAGAATTAGAAGCAC
T E N E I K I L C L S S R E I F L N Q P I L L E L E A
CAATAAAAATATGTGGAGATATCCATGGACAGTTTATGATTTGTTAAGGTTATTTGAATATGGTGGATTTCCACCCGATGCA
P I K I C G D I H G Q F Y D L L R L F E Y G G F P P D A
AATTATCTATTTTTAGGttaaataaaagaaaaaataatataatataatataatataatataatataaagttggcatataatc
N Y L F L
ttttatacatgtacatatTTTTTTgTtccTTTTtatagGTGATTATGTGGATAGAGGAAAACAAAGTTTAGAAACCATTGT
G D Y V D R G K Q S L E T I C
TATTATTAGCATATAAAAATAAAAATATCCTGAAAATTTTTTTTTATTAAAGAGGTAACCACGAATGCCTTCAATAAATAGAATA
L L L A Y K I K Y P E N F F L L R G N H E C A S I N R I
TACGGATTCTATGACGAATGTAAGAAGATATAGTGTGAAATTATGGAAAACATTTATTGATTGCTTTAATTGCTTACCTGT
Y G F Y D E C K R R Y S V K L W K T F I D C F N C L P V
GGCAGCTATTATTGACGAAAAATTTTTTGTATGCATGGTGGTTTATCACCTGAATTAATAATGGAACAAAATAAGGAAAA
A A I I D E K I F C M H G G L S P E L N N M E Q I R K
TAACTAGGCCTACTGATGTTCTGATAATGgtatTTTgacaaatgatatgaataaatagaatgaggaaaatattaagtatata
I T R P T D V P D N
taatgTgTgtaaatgTTTTTTTTTTTTTTTTTTTTTTTTTTTcattatattatgccccataaacaatatataatata
tatataatataaaagtatatgtatatgtgTtatttattttttttttttttttttagatagGTTTATTATGT
G L L C
GATTTATTGTGGTCTGATCCAGAAAAAGAAATTAATGGGTGGGGAGAAAAATGATCGAGGAGTTTCTTTTACCTTTGGTCAAGA
D L L W S D P E K E I N G W G E N D R G V S F T F G Q D
TGTTGTTCAACTTTTTTAAGAAAAACACGAATTAGATTTAATATGCAGGGCACATCAGgtaatatattaataaatgTTTTTT
V V H N F L R K H E L D L I C R A H Q
TTTTTTTTTTTTTtattctatatgactactaataatgtgTtTtTtgaataatgTgattgTtTtTtgaattgataTTTTTat
aagaatatataatataatataatataatataatataatgtatatattcttattataacatttTgTgaattTTTTTTTTctaa
atagTTCGTGGAGGATGGATATGAATTTTTTGCAAAGCGCCAATTAGTTACATTATTTTCTGCTCTAATTATTGTGGAGAGT
V V E D G Y E F F A K R Q L V T L F S A P N Y C G E
TTGATAATGCCGGTCAATGATGAGTGTGACGAGACATTAATGTGTTTCGTTTCAAgtatgatataagaaaatgaatataaa
F D N A G A M M S V D E T L M C S F Q
taaataaataaataaataatataatataatataatagacaagaatgTtTTTTtTtTtcatattttatataTTTTTtattttt
cacgTttacatttatttcttctttagATTTTTAAACAGTGGAAAAAAGAAAGCAGCAAATTAABCDEFGHI
I L K P V E K K K A A N -

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Figure 1

PfPP1 gene structure. The exon and intron sequences of PfPP1 gene are shown in capital and small letters, respectively. Underlined primers were used in RT-PCR to amplify the PP1 ORF, and have been described under Results. The amino acid sequence of PfPP1 is in single-letter codes below the coding sequence.

shortest PP1 known to date, and lacks a short proline-rich stretch at the C-terminal end (Fig. 2).

Expression and catalytic properties of PfPP1

In order to characterize the PfPP1 cDNA we subcloned it into pET-15b such that the protein is expressed with an N-terminal (His)₆-tag. As shown in Fig. 3, a protein band of approximately 37,000 Mr was produced from the pET-15b-PfPP1 clone, which is in agreement with the predicted molecular weight of 34,904 of PfPP1 and roughly another 2,000 added for the (His)₆ region of pET-15b (Novagen: Madison, WI). The protein specifically reacted with a monoclonal anti-His antibody and also with a pol-

yclonal antibody against full-length human PP1 (Transduction Laboratories: Lexington, KY). The recombinant protein was purified through nickel-chelation chromatography and tested for phosphatase activity. It dephosphorylated the small substrate pNPP, as well as histone, labelled at Ser residues. Interestingly, it also showed decent activity on a Tyr-phosphorylated synthetic peptide. The V_{max} values ($\mu\text{mol Pi}$ liberated / mg enzyme/ min) against these three substrates (pNPP, phosphoserine-histone, phosphotyrosine peptide) were, respectively: 12 ± 2, 8 ± 1, and 2 ± 0.5. An equivalent protein fraction, obtained from *E. coli* containing vector alone (without insert), showed no activity against any of these substrates. It has

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Plasmodium  --MALEIDIDNVISKLIEVRGTRPGKNVNLTEENEIKILCLSREIFLNQPIILLELEAPIK 58
Human       MSDSEKLNLDISIIGRLLEVQGSRPGKNVQLTEENEIRGLCLKSREIFLSQPILLELEAPLK 60
              *.:*.:*.:*:*:*:*:*:*:*:*:*:*:*:*.:*:.*****.*****:*
              *.:*.:*.:*:*:*:*:*:*:*:*:*:*:*:*.:*:.*****.*****:*

Plasmodium  ICGDIHGQFYDLLRLFEYGGFPDANYLFLGDYVDRGKSLETICLLLAYKIKYPENFFL 118
Human       ICGDIHGQYDILLRLFEYGGFPESNYLFLGDYVDRGKSLETICLLLAYKIKYPENFFL 120
              *****:*****:*****:*****:*****:*****

Plasmodium  LRGNHECASINRIYGFYDECKRRYSVKLWKTFTIDCFNCLPVAAIIDEKIFCMHGGLSPEL 178
Human       LRGNHECASINRIYGFYDECKRRYNIKLVKTFIDCFNCLPIAAIVDEKIFCCHGGLSPDL 180
              *****:*****.:***** *****:***:***** *****:*

Plasmodium  NNMEQIRKITRPTDVPDNGLLCDLLWSDPEKEINGWGENDRGVSTFTFGQDVVHNFRLKHE 238
Human       QSMEQIRRIMRPTDVPDQGLLCLDLWSDPKDVQGWGENDRGVSTFTFGAENVAKFLHKHD 240
              ..*****:* *****:*****:*. *****:***:**:*

          β12  loop  β13
Plasmodium  LDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPVE 298
Human       LDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPAD 300
              *****:*****:*****:*****:*****:*****:*****:

Plasmodium  KKK-----AAN----- 304
Human       KNKGYGQFSGLNPGGRPIPPR--NSAKAKK----- 330
              *.*
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Figure 2

PfPP1 sequence comparison. The predicted sequences of *Plasmodium* PP1 (this study) and human PP1 alpha (P08129) catalytic subunits were aligned using the CLUSTALW program at the European Bioinformatics Institute (EMBL) server, and later refined by visual inspection. The amino acid residue numbers are shown on the right. Residues are marked as: non-conservative replacement (.); conservative replacement (.), and identical (*). Residues important in I-2 interaction are highlighted in gray: E52, E54; D164, E165, and K166.

been recently shown that rabbit PP1, expressed in *E. coli*, also possessed Tyr phosphatase activity. In contrast, native PP1 isolated from rabbit muscle or expressed in Sf9 insect cells contained only Ser/Thr phosphatase activity but no Tyr phosphatase activity [24]. It was concluded that this apparent difference might be due to subtle changes in protein folding in eukaryotic versus prokaryotic cellular environments. It remains to be seen whether this is a general feature of all PP1.

To obtain biochemical evidence for the identity of the recombinant PfPP1, we tested the effect of specific phosphatase inhibitors and selected mutations on the phosphatase activity. Mutation of Asn122 to Asp by site-directed mutagenesis destroyed the phosphatase activity, confirming the essential role of this residue of PP1 in catalysis [19]. PfPP1 was inhibited by NaF, inorganic orthophosphate, and pyrophosphate at respective IC₅₀ values of 2.5 mM, 10 mM, and 90 μM (data not shown). Similar

values were recently obtained for *Arabidopsis* PP1 [8]. PfPP1 was also inhibited by tautomycin, I-1, I-2, and OA with IC₅₀ values of 0.8, 400, 7, and 100 nM, respectively (Fig. 4). These values are comparable to those obtained with various PP1 isoforms recombinantly expressed in *E. coli* [25,29]. The sensitivity of PfPP1 to these natural toxins is consistent with the fact that the loop sequence between the β12 and β13 regions plays a direct role in binding these toxins [23,26], and this sequence is entirely conserved in PfPP1 (Fig. 2). Recently, a few additional residues that are closer to the N-terminus in the PP1 sequence have also been shown to be important in the interaction with I-2 [24]; in PfPP1 numbers, these residues are: E52, E54, and D164, E165, K166 (Fig. 2). In yeast PP1 (Glc7p), the double mutant E52A/E54A and the triple mutant D164A/E165A/K166A showed IC₅₀ values for I-2 that were respectively 8 and 300 times the wild type enzyme values [24]. As shown in Fig. 4, a similar loss of inhibition by I-2 was also observed when the corresponding muta-

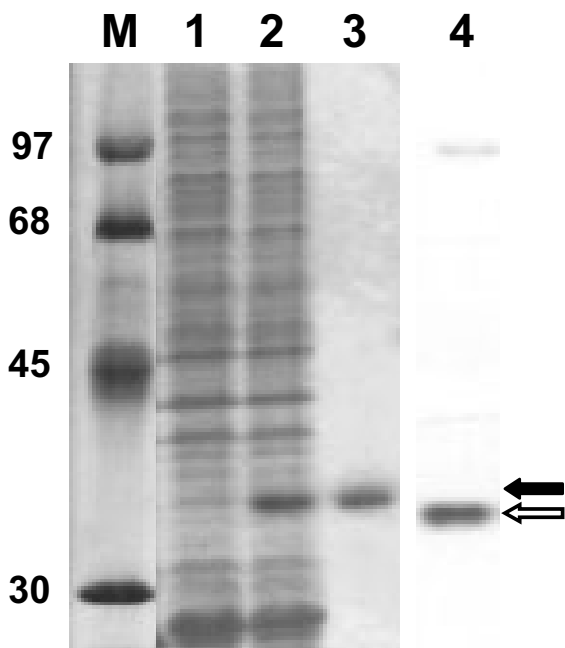


Figure 3

Recombinant expression of PfPP1 in bacteria. The following proteins / extracts were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R250: approximately 30 μ g (protein) of total extract [14] of IPTG-induced *E. coli* BL21(DE3) containing the RIG plasmid and pET-15-PfPP1 (lane 2) or pET-15 without insert (lane 1); 4 μ g of the purified recombinant (His)₆-tagged PfPP1 (lane 3). Lane 4 shows an immunoblot in which 80 μ g of 100,000 \times g extract of Pf [10] was probed using a PP1 antibody described under Materials and Methods. Parasitic PfPP1 and the recombinant His-tagged PfPP1 bands are indicated by open and closed arrowheads, respectively. Protein markers (lane M) are indicated by Mr in thousands.

tions were introduced into PfPP1, although they did not affect the catalytic activity (specific activity) (data not shown). Together, these results provide experimental confirmation of the catalytic identity of PfPP1.

Expression of native PfPP1

As mentioned earlier, inhibition studies using OA and calyculin A suggested the existence of a PP1-like activity in *P. falciparum* extracts [15]. However, our attempts to purify the native PfPP1 enzyme by chromatographic procedures resulted in only small amounts of activity, probably due to rapid inactivation during fractionation. To determine if PfPP1 is expressed in *Plasmodium*, we have, therefore, taken an immunological approach. First, cell-free extracts of different erythrocytic stages of *P. falciparum* were subjected

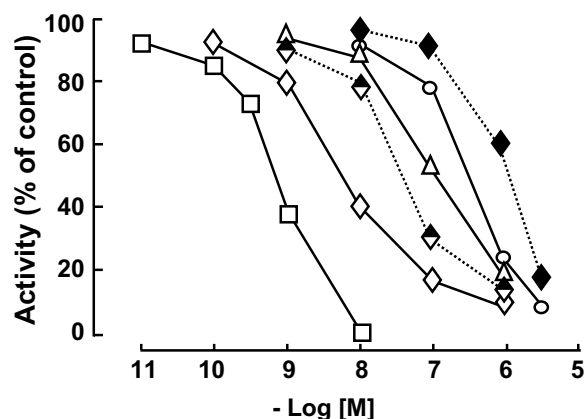


Figure 4

PfPP1 dose response to inhibitors. Inhibition assays for recombinant PfPP1 were performed using ³²P-phosphorylase α as substrate essentially as described [14]. The inhibitors and the symbols are: tautomycin (square), inhibitor-2 (diamond), OA (triangle), and I-1 (circle). The half-closed and fully closed diamonds represent I-2 against the double mutant (E52A/E54A) and triple mutant (D164A/E165A/K166A) enzymes, respectively. Activities are expressed as percentage of the inhibitor-free reaction. As shown, the X-axis represents negative logarithm of molar concentration of the inhibitors.

to Western blot using a monoclonal antibody that was raised against nearly full-length human PP1 and showed a broad species specificity (see Materials and Methods). As shown in Fig. 5A, a major band of the expected size of 35 k was observed in all stages including gametes. A peptide antibody, specific for human PP1 (Materials and Methods), did not detect the band. The gametogenesis was confirmed by the appearance of Pf27, an early gamete-specific protein [30]. Having demonstrated that the monoclonal antibody was specific for PP1, we used it in an attempt to inhibit PfPP1 activity *in vitro*. At optimal concentrations, the antibody almost completely inhibited (90% inhibition) recombinant PfPP1 (including its Tyr phosphatase activity), and also inhibited the phosphatase activity of the *P. falciparum* extract by about 70% (data not shown). Under the same conditions, the human-specific antibody had no effect. Finally, it has been shown that affinity resins containing immobilized microcystin specifically bind toxin-sensitive phosphatases such as PP1 and PP2A [31]. Thus, we passed soluble cytosolic Pf extract through microcystin-Sepharose, and the bound proteins were analyzed on SDS-PAGE followed by immunoblot using the anti-PP1 antibody. The blot revealed that the 35 kDa PfPP1 polypeptide indeed specifically bound to microcystin (Fig. 5B), correlating the antigenic reactivity of PfPP1 with its affinity for the toxin. Pre-incubation of the

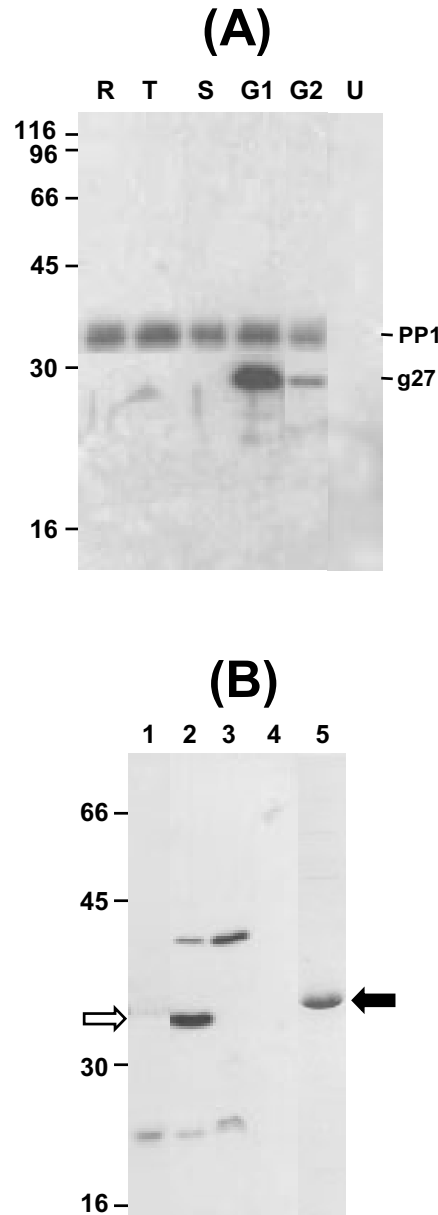


Figure 5

Constitutive expression of parasitic PfPPI. (A) Western blot: Total protein (80 μ g) from the ring (R), Trophozoite (T), schizont (S), and early (G1) and late (G2) sexual stages of Pf were probed with a mixture of anti-PPI and anti-Pfg27 antibodies as described [14]. (B) Microcystin-sepharose chromatography: About 500 μ g of the following extracts was subjected to microcystin affinity chromatography and the bound proteins analyzed by Western blot using PPI antibody: extract of uninfected RBC processed identically (lane 1); Pf extract (lane 2); Pf extract pre-incubated with 1 μ M microcystin-LR at room temperature for 5 min (lane 3); unbound fraction (a double-pass flow-through from the column) (lane 4). Recombinant His-tagged PfPPI is displayed in lane 5 for comparison. The native and recombinant PPI bands are marked by open and closed arrows, respectively. Sizes of protein standards are indicated on the left. Two non-PPI proteins are also seen in the blot in panel B. The ~25 kDa band (common in lanes 1, 2, and 3) is evidently a RBC protein. The ~40 kDa band (lanes 2, 3), on the other hand, is a *Plasmodium* protein, since it is absent in the RBC fraction. We speculate that these proteins non-specifically bound to the Sepharose matrix, since they could not be competed out by microcystin (lane 3).

extract with microcystin specifically prevented binding of PfPP1 to the column.

Generation of phenotypic PfPP1 mutants by PTGS using RNAi

RNA interference (RNAi), mediated by short interfering double-stranded RNA molecules (siRNA or dsRNA), is now recognized as a major mechanism of post-transcriptional gene silencing (PTGS) in essentially all eukaryotes [16]. Recently, the technique has been successfully applied to cultured mammalian cells, whereby introduction of 21-nucleotide long synthetic dsRNA molecules corresponding to specific mRNA sequences effectively and specifically degraded the cognate mRNAs and abrogated the expression of the corresponding proteins [17,18]. This prompted us to test a similar approach to knockdown PfPP1 function in the erythrocytic *P. falciparum* stages. Our initial attempts with standardized transfection procedures using OligofectAMINE (Life Technologies: Bethesda, MD) with the dsRNA did not produce an appreciable loss of PfPP1. We then resorted to the electroporation procedure originally developed for DNA transfection in *Plasmodium* by Welles and co-workers [32], as detailed under Materials and Methods. A representative set of results shown in Fig. 6 clearly demonstrates loss of PfPP1 by the RNAi procedure, while the control PP2A was not significantly affected. Loss of PfPP1 resulted in concomitant inhibition of parasite growth as evidenced by the drastic reduction in ³H-hypoxanthine incorporation. These results suggest that PfPP1 plays an essential role in *Plasmodium* replication. The facts that the parasite culture was asynchronous, i.e., contained all three major stages (ring, trophozoite, and schizont) (data not shown) and that the effect of dsRNA was severe, suggest that PfPP1 is required for cell cycle progression at all stages of the parasite. This is further supported by the expression of PP1 protein in all the parasitic stages (Fig. 5). Taken together, this is consistent with the established role of PP1 in eukaryotic DNA synthesis and cell cycle progression, as discussed below.

PP1 is one of the major protein phosphatases found in all eukaryotic cells. The activity of the catalytic subunit of PP1 is controlled by its interaction with a large number of regulatory subunits, many of which also target it to specific subcellular compartments [7,24,33]. The major ones include the glycogen-targeting subunits (G_M , G_L) [34], myofibrillar-targeting subunit (M_{110}) [34], nuclear inhibitor of PP1 (NIPP-1) [35], PP1 nuclear targeting subunit (PNUTS) [36,37], mitosis-regulating subunit Sds22 [38–40], ribosomal protein L5 [41] and small cytosolic inhibitory proteins, I-1, I-2, and DARRP-32 (Dopamine and cAMP-regulated phosphoprotein, Mr 32,000) [7,23,25]. The physiological role of many of these interactions has been revealed in recent studies. A temperature-sensitive mutant of the yeast PP1 (Glc7), for example, exhibits a

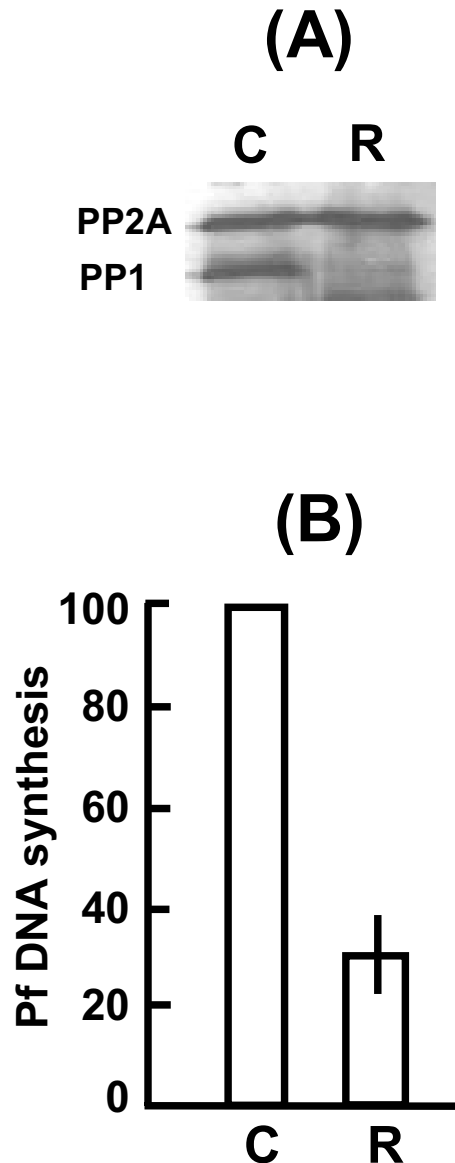


Figure 6

Inhibition of parasite growth and abrogation of PfPP1 expression by interfering dsRNA. The dsRNA sequences, and electroporation procedure have been described under Materials and Methods. Infected RBC were transfected with dsRNA against PfPP1 (R) or luciferase (C). Western blot (A) shows loss of PfPP1 by the dsRNA, but no effect on the control PfPP2A. The PP1 monoclonal and PP2A peptide antibodies have been described in Materials and Methods. Hypoxanthine incorporation assay (B) shows an approximately 70% inhibition of parasitic DNA synthesis in PP1-depleted cells, compared to the luciferase antisense-treated control.

block in the M phase of the cell division cycle, and elevated expression of Sds22 suppresses this defect [38]. Structure-function analysis of recombinant mammalian PP1 has recently begun to map specific residues involved in interaction with other molecules. Interestingly, all these residues are conserved in PfPP1, and a few examples are presented here. Co-crystal structure of PP1 and a G_M peptide [42], as well as mutagenesis studies [43] of the yeast PP1 ortholog, Glc7, have demonstrated a role of specific PP1 residues in PP1- G_M interaction. In the PfPP1 sequence, these residues are: I167, L241, F255, L264, C289, and F291 (Fig. 2). As mentioned, the β 12-loop- β 13 region, important in interaction with natural toxins [23], is fully conserved in PfPP1 (Fig. 2). Two residues, recently shown to be important for interaction with DARPP-32 [22], are also conserved in PfPP1; these are: Met288 and Cys289 (Fig. 2). We have provided experimental evidence of an important role of a number of invariant residues of PfPP1 in the interaction with I-2 (Fig. 2 and 4). Based on such overwhelming conservation of functionally important residues, we propose that orthologs of many of these PP1-interacting proteins may also exist in *Plasmodium* and function in similar roles. The use of recombinant PfPP1 and protein-protein interaction techniques should aid in characterizing these physiological regulatory subunits of PfPP1. Our ability to generate PP1-deficient *P. falciparum* parasites will allow us to study the "mutant" phenotype in further detail and understand the role of this highly conserved phosphatase in malarial biochemistry and pathophysiology. These studies are in progress.

As mentioned earlier, Li and Baker [12] described a putative phosphatase DNA sequence in *Plasmodium*, the mRNA of which was detected in the sexual stages of the parasite. The predicted protein, named PP α , was 889 amino acid long, and contained a unique N-terminal extension of about 500 amino acids. The C-terminal 345 amino acids, containing the putative catalytic domain, had 5 unique peptide stretches that were called "inserts". When these inserts were omitted from the alignment, the rest of the sequence showed significant similarity with PP1 phosphatases [12]. Clearly, further studies are needed to identify the PP α protein and characterize its potentially interesting identity.

While our manuscript was being written, McRobert and McConkey [44] achieved similar success in using the RNAi strategy to ablate dihydroorotate dehydrogenase (DHODH) of *P. falciparum*. Although the protein level was not directly monitored, the loss of DHODH mRNA was confirmed by RT-PCR. This resulted in inhibition of parasite growth, consistent with the role of DHODH in pyrimidine biosynthesis, essential for parasite DNA replication. These authors introduced the double-stranded RNA by electroporation also, using conditions very simi-

lar to ours. Thus, although the exact mechanism of the RNA uptake remains to be elucidated, the electroporation procedure must have allowed the dsRNA to traverse the various erythrocytic and parasitic membranes to enter the parasitic cells.

The success in ablating PfPP1 is particularly gratifying to us on a number of accounts. As mentioned, PP1 is a highly conserved enzyme, and toxin-sensitive Ser/Thr phosphatase activities are also found in erythrocytes [15]. Thus, use of PP1 inhibitors, such as okadaic acid or tautomycin (Fig. 4) would affect the parasite as well as the host, making conclusions difficult. The dsRNA, in contrast, is known to be extremely specific for its intended target, such that a single nucleotide mismatch prevents its action [17]. This has allowed us to create specific phenotypic loss of PfPP1. Moreover, RNAi will permit the ablation of essential gene products, at any time point in infection, or on a desired parasitic stage following synchronization. Lastly, traditional genetic manipulation in eukaryotes, including the *Apicomplexa*, is a relatively difficult and elaborate procedure [45,46]. Thus, we believe that the RNAi strategy will become a powerful and convenient tool in *Plasmodium* functional genomics, particularly in the studies of phylogenetically conserved signalling molecules.

Conclusion

P. falciparum contains a PP1 protein phosphatase that is virtually identical to its orthologs in other species in both sequence and biochemical properties. Based on the established physiological role of PP1 in other organisms such as mammals and yeast, PfPP1 may regulate a variety of parasitic pathways, including glycogen metabolism, glucose repression, and cell cycle progression. Indeed, the successful use of RNA interference to ablate PfPP1 confirms its essential role on parasitic growth. The catalytic subunit of PfPP1 is expressed in all the erythrocytic stages of the parasite and is specifically inhibited by mammalian physiological inhibitors, inhibitor-1 and inhibitor-2. Thus, an in-depth study of PfPP1 and its interacting subunits may shed light on the regulation of the relevant pathways in this clinically important family of parasites.

Materials and methods

Materials

Histone and the catalytic subunit of PKA were purchased from Sigma (St. Louis, MO), and rabbit I-2, the *v-abl* protein tyrosine kinase, and its peptide substrate EAIYAAP-FAKKK were from New England Biolabs (Bedford, MA). Okadaic acid (OA) and recombinant I-1 [22] were kind gifts from R. Honkanen and S. Shenolikar (Duke University), respectively. The monoclonal anti-PP1 antibody was raised against a 25.6 kDa fragment of human PP1 α (residue 5–226), and was purchased from Transduction Laboratories (Lexington, KY). This antibody reacts with all

mammalian and avian PP1 tested, but does not react with other PP classes. The other PP1 antibody (a kind gift from R. Honkanen) was specific for human PP1, and was raised in rabbit against a synthetic peptide corresponding to the last 13 residues of human PP1 (PITPPRNSAKAKK; Fig. 2). The anti-PfPP2A antibody was raised in rabbit against the peptide MLIFKDTSPDRNSIKN, corresponding to residue 285–300 of the previously described PfPP2A [10]. The RIG plasmid was kindly provided by W. Hol (University of Washington, Howard Hughes Medical Institute) [47]. Monoclonal antibody against the early sexual-stage *P. falciparum* antigen Pfg27 [30] was a generous gift from N. Kumar (Johns Hopkins University, Baltimore, MD). Microcystin-coupled sepharose was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Amplification, cloning, and mutagenesis of PfPPI cDNA

Total RNA was isolated from asynchronous *P. falciparum* 3D7 cells grown in human A-positive erythrocytes essentially as before [48,49]. Various pairs of primers were designed on the basis of the relevant sequences of chromosome 14. T_m values of the primers were in the range of 65–72°C in order to achieve high specificity in reverse transcription (RT) as well as PCR. RT reaction was carried out at 60°C for 1.5 hrs using the C. therm. polymerase kit (Roche Molecular Biochemicals, Cat. No. 2016311). The RT reaction was incorporated into PCR, carried out using a mixture of Taq (Roche) and Pfu (Stratagene) polymerases (20:1) to ensure high fidelity [50]. Elongation in PCR was performed at 62°C. The various products were gel-purified and cloned in pGEM-T by "TA cloning" (Promega, WI). The clones were initially screened by restriction analysis and finally confirmed by sequencing.

All site-directed mutagenesis were performed using the megaprimer procedure [51], and the mutations were confirmed by sequencing. DNA sequencing was carried out by cycle sequencing using the PRISM Big Dye Terminator sequencing kit and AmpliTaq DNA polymerase (Perkin-Elmer, Division of PE/ABI).

Expression and assay of recombinant PfPPI phosphatase

Growth and induction of *E. coli* BL21(DE3) containing pET-15b-PfPPI and the RIG plasmid were carried out using procedures described earlier [14,48], except that the culture was grown at 18°C in the presence of 2 mM $MnCl_2$, and IPTG concentration was lowered to 0.4 mM. The (His)₆-tagged PfPPI expressed from pET-15b-PfPPI was purified through Ni²⁺-chelation chromatography [14] as described by the manufacturer (Novagen), with 1 mM $MnCl_2$ being present in all the buffers. The imidazole-eluted His-tagged PfPPI was dialyzed against 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 25% glycerol, 1 mM DTT (buffer A), and stored in small portions at -80°C.

Phosphatase activities were assayed essentially as described [14,19,52]. Unless otherwise mentioned, 80 µl reactions contained 2 mM of $MnCl_2$ and requisite amount of recombinant enzyme in buffer A. Where mentioned, OA was directly added to the reaction. When I-2 was used, it was pre-incubated with PfPPI at 32°C for 30 min. I-1 was prephosphorylated by PKA in a standard kinase reaction containing 200 µM γ -thiophosphorylated ATP. ³²P-labelled histone was prepared by phosphorylation with PKA in the presence of γ -³²P [ATP] essentially as described, followed by removal of the free ATP by gel filtration [19,52]. The resultant phosphohistone is exclusively phosphorylated at Ser residues [53]. ³²P-labelled peptide EAI(Yp)AAPFAKKK, phosphorylated at the single Tyr residue by pp43^{v-abl} kinase, was prepared essentially as described [19,52]. Phosphatase reactions were initiated by the addition of the substrate. The liberated ³²P was quantitated by a phosphomolybdate extraction assay as described previously [14]. Reactions were followed with time, and results were corrected by subtraction of the corresponding values from an enzyme-free reaction.

Analysis of native PfPPI

P. falciparum 3D7 was grown on A-positive human erythrocytes in the presence of homologous serum as described earlier [48,49]. When needed, cultures were synchronized in two steps [49,54]: (i) schizonts were purified by flotation over 65% (v/v) Percoll (Pharmacia) followed by incubation with fresh erythrocytes (5% haematocrit); (ii) the cultures were then left to mature into rings and treated with 5% D-sorbitol for 15 min at 37°C. The purity of individual stages was greater than 95% as confirmed by microscopic observation of a stained thin smear of the culture. Sexual stage parasite was generated as described [30].

Transfection by inhibitory dsRNA

The following 21-mer RNA molecules, corresponding to the underlined sequence in Fig. 1, were synthesized as described [18] and deprotected according to the manufacturer's protocol (Dharmacon Research, Lafayette, CO):

Sense: (5') GAGGUAACCACGAAUGCGCdTdT (3')

Antisense: (5') GCGCAUUCGUGGUUACCUCdTdT (3')

The negative control luciferase RNA was the same as the double-stranded GL3 RNA described previously [17]. The RNAs were annealed *in vitro* to form double-stranded RNA (dsRNA) [18] and electroporation was carried out essentially as described [32]. In brief, 3 µg dsRNA in 800 µl of incomplete cytomix [32] was added to infected RBC (at 10–15% parasitemia), and electroporation was performed using a Bio-Rad Gene Pulsar unit at settings of 200 Ω, 2 kV, and 25 µF. Control cells were identically electro-

porated without RNA. The cells were then grown in 12-well plates in triplicate wells, and measurement of ³H-hypoxanthine incorporation was carried out at 24 hr post-electroporation using standard procedures [49], except that parasites were liberated with saponin, pelleted, and counts in the pellet measured following solubilization. Parasites from unlabelled but otherwise identical cultures were analyzed in Western blot using a mixture of anti-PP1 and anti-PfPPP2A antibodies (see Materials and Methods).

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