

Research article

A novel tetratricopeptide repeat (TPR) containing PP5 serine/threonine protein phosphatase in the malaria parasite, *Plasmodium falciparum*

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Abstract

Background: The malarial parasite, *Plasmodium falciparum* (Pf), is responsible for nearly 2 million deaths worldwide. However, the mechanisms of cellular signaling in the parasite remain largely unknown. Recent discovery of a few protein kinases and phosphatases point to a thriving reversible phosphorylation system in the parasite, although their function and regulation need to be determined.

Results: We provide biochemical and sequence evidence for a protein serine/threonine phosphatase type PP5 in *Plasmodium falciparum*, and named it PfPP5. The 594-amino acid polypeptide was encoded by a 1785 nucleotide long intronless gene in the parasite. The recombinant protein, expressed in bacteria, was indistinguishable from native PfPP5. Sequencing comparison indicated that the extra-long N-terminus of PfPP5 outside the catalytic core contained four tetratricopeptide repeats (TPRs), compared to three such repeats in other PP5 phosphatases. The PfPP5 N-terminus was required for stimulation of the phosphatase activity by polyunsaturated fatty acids. Co-immunoprecipitation demonstrated an interaction between native PfPP5 and Pf heat shock protein 90 (hsp90). PfPP5 was expressed in all the asexual erythrocytic stages of the parasite, and was moderately sensitive to okadaic acid.

Conclusions: This is the first example of a TPR-domain protein in the *Apicomplexa* family of parasites. Since TPR domains play important roles in protein-protein interaction, especially relevant to the regulation of PP5 phosphatases, PfPP5 is destined to have a definitive role in parasitic growth and signaling pathways. This is exemplified by the interaction between PfPP5 and the cognate chaperone hsp90.

Background

On the basis of sequence homology and similarity of three-dimensional structures, phosphoprotein phosphatases (PPases) have been classified into three fami-

lies designated PPP, PPM, and PTP [reviewed in [1–3]. The PPP and PPM families are comprised of phosphoserine- and phosphothreonine-specific enzymes whereas the PTP family consists of phosphotyrosine-specific and

dual-specificity enzymes [4]. The major members of the PPP family are PP1, PP2A, and PP2B (calcineurin) class of phosphatases. Protein phosphatase 5 (PP5), a newer member of the PPP family, differs from the other Ser/Thr phosphatases in that it contains regulatory and (sub)cellular targeting functions within a single polypeptide [5–7]. While its catalytic core exhibits strong similarity to those of the other members of this family, its N-terminus consists of three tetratricopeptide repeats (TPRs) that are unique to the PP5 class. TPR domains consist of a series of antiparallel amphipathic α helices that bundle together through hydrophobic interactions to form a cradle-shaped groove, postulated to be involved in binding a number of proteins of regulatory importance such as heat shock protein 90, a major cellular chaperone [8–10].

The *Apicomplexan* family of parasites, exemplified by *Plasmodia*, are major disease agents of humans. As the causative agent of malaria, *Plasmodia sp.* alone infects about 300 million people globally and results in an annual death toll of nearly 2 million [11]. *Plasmodium falciparum* (Pf) is the most virulent of all and causes fatal cerebral malaria. Because of the continual emergence of drug-resistant parasites throughout the world, a need for a fundamental knowledge of the signaling pathways of the parasite has been recognized. In the recent past, this has led to the identification of a number of *Plasmodium* protein phosphatases, some putative [12,13], others experimentally demonstrated [e.g., [14–16]]. Most of these phosphatases resembled the classical mammalian PP1, PP2A, PP2B and PP2C enzymes [12,14,16], and some were potentially novel Ser/Thr phosphatases [13,15,16].

In this report, we describe the cloning and characterization of a novel PP5 phosphatase from Pf (PfPP5) that contains an unusually long N-terminal extension that contained four putative TPR motifs and played an important role in fatty acid-mediated activation of the enzyme. The structural and biochemical properties of PfPP5 described herein are hallmarks of the PP5 class, and thus establish PfPP5 as a likely player in parasitic signal transduction, and hence a potential target for anti-malarial drug design.

Results

Identification of PfPP5 cDNA and gene

To identify new Ser/Thr phosphatases of Pf, we have recently undertaken a PCR-based approach. At first, we made degenerate deoxyoligonucleotide primers corresponding to the conserved peptide sequences GDXHGQ and GDXVDRG of PPs [17]. An approximately 120 bp PCR product, obtained by using these primers with the Pf 3D7 genomic DNA as template, was cloned in the pGEM-T vector (Promega) using the T/A cloning strate-

gy. Sequencing of individual clones revealed a variety of potential PPP sequences in Pf, one of which was an exact match with a putative open reading frame (ORF) on Pf chromosome 13 in the sequence database of the Sanger Center (Accession No. ALO49185). Comparison of the predicted primary structure of the protein clearly revealed its similarity with known members of the PP5 family, including the presence of multiple TPR domains (Fig. 1 and 2). Specific primers were then made against this ORF and the 1578 base pair gene was cloned and confirmed by sequencing. The same set of primers were then used to amplify the PfPP5 cDNA sequence from a poly(A)-enriched mRNA population of Pf strain Dd2. This sequence fully matched the genomic Sanger sequence, suggesting the lack of introns in the PfPP5 gene.

Recombinant expression of PfPP5

To express the putative PfPP5 protein, we subcloned it in the bacterial expression vector pET-15b and introduced the plasmids into *E. coli* BL21(DE3). As shown in Fig. 3, when induced with IPTG, the pET-15b-PfPP5 clone produced a polypeptide of approximate Mr 70 k (lane 2), which is in excellent agreement with the calculated MW of 69,139, considering that pET-15b adds a (His)₆ tag (about 2 kDa) to the N-terminus of the recombinant protein. The presence of the (His)₆ tag could be confirmed by anti-His antibody in immunoblot (data not shown). The His-tagged PfPP5 was subsequently purified through standard nickel-chelation chromatography (Fig. 3, lane 5). Using ³²P-labeled histone as substrate under standard assay conditions (in absence of any activator), the specific activity of the recombinant PfPP5 was measured to be 198 ± 25 nmoles of ³²P/min/mg enzyme.

The deletion mutant, Δ TPR-PfPP5, was similarly cloned by PCR using a primer corresponding to the internal sequence, and purified by nickel chelation affinity chromatography. The deletion mutant starts with Met-273, as described in Fig. 1.

Identification of native parasitic PP5

To identify whether PfPP5 is actually expressed in the parasite, cell-free extracts of the asynchronous erythrocytic stage parasites were subjected to standard chromatographic procedures that had been previously optimized for human PP5 [18]. Studies of mammalian PP5 have revealed that the TPR domain might act as an auto-inhibitory domain of the phosphatase activity, and that tryptic digestion of PP5 causes loss of the TPRs and concomitant stimulation of activity [19–21]. As described under Materials and Methods, the purification was in fact monitored by assaying for a trypsin-activated phosphatase with the assumption that PfPP5 may behave like mammalian PP5 in this regard [18,20,21]. As mentioned, the activity peaks always coincided with a

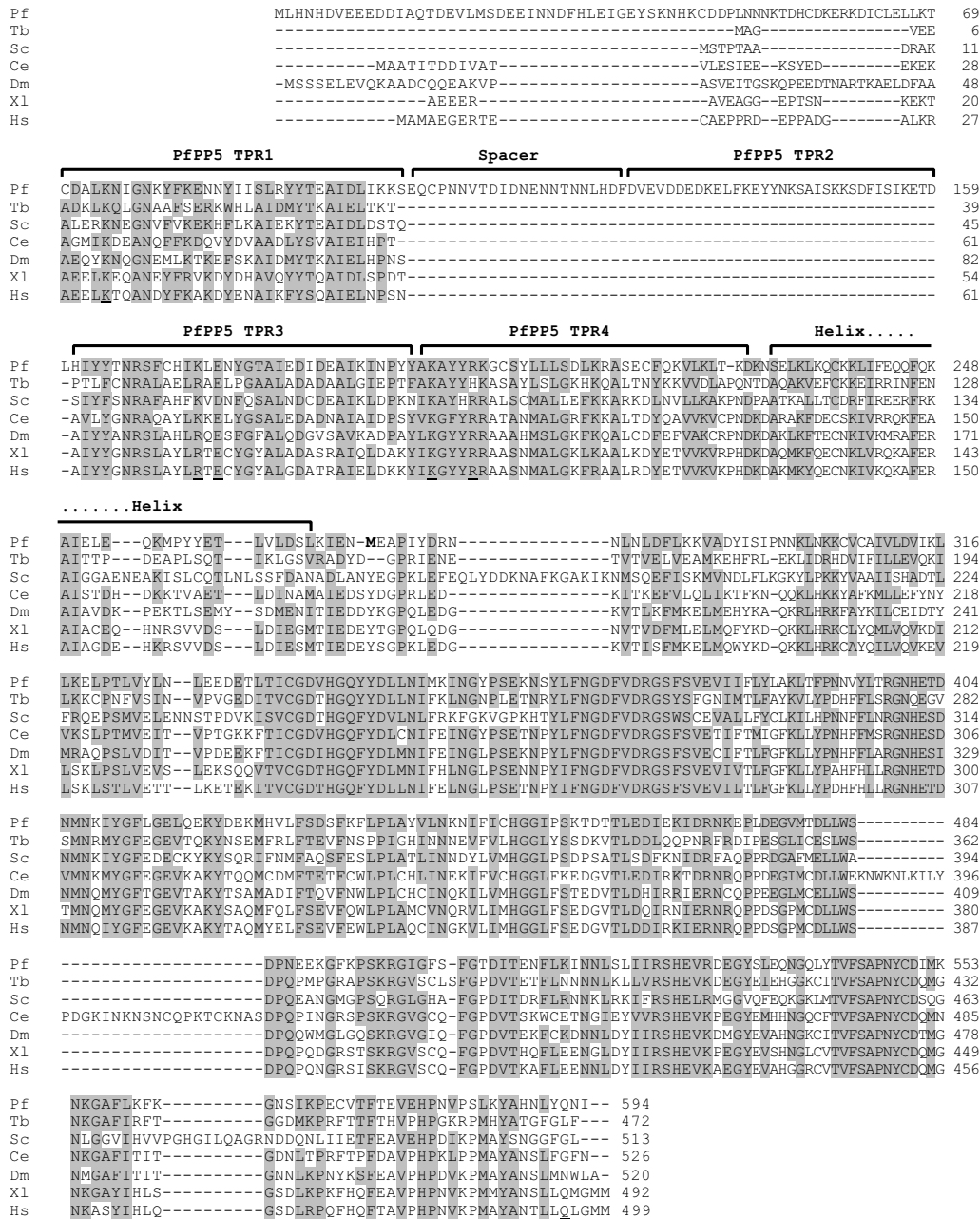


Figure 1
 Comparison of the primary structure of PfPP5 with representative orthologs. The predicted sequences of PP5 from the following organisms (abbreviation and accession numbers in parenthesis) were aligned using the CLUSTALW multiple alignment program at the European Bioinformatics Institute (EMBL) server, and later refined by visual inspection: *Plasmodium falciparum* (Pf; AL049185); *Trypanosoma brucei* (Tb, AAG40278); *S. cerevisiae* (Sc, P53043); *C. elegans* (Ce, CAB60937); *Drosophila melanogaster* (Dm, AAF54438); *Xenopus laevis* (Xl, AAB70574); *Homo sapiens* (Hs, AAD22669). The numbers of amino acid residues are shown on the right. The four proposed TPR domains of PfPP5, the spacer, and a helix are so indicated. Specific amino acid residues important in autoinhibition and interaction with hsp90 with mammalian PP5 are underlined on the bottom (human) sequence. Residues that are identical or conservative replacements (T, S; A, M, L, I, V; D, E; R, K; Q, N; F, Y) in at least 4 sequences are highlighted in gray. The TPR-deletion mutant starts at Met-273 (bold).

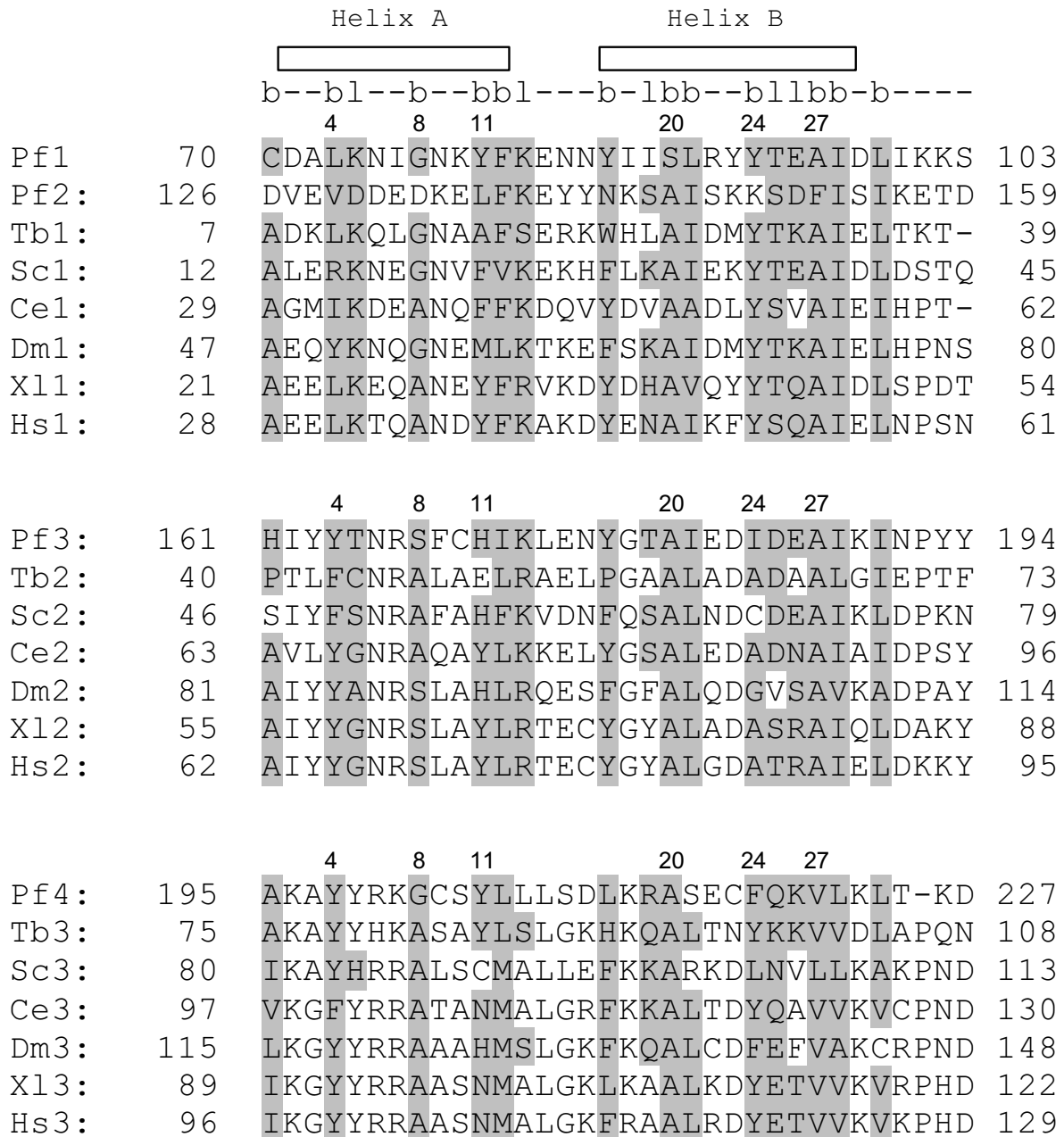


Figure 2

Comparison of representative PP5 TPR domains. The TPR domains of the PfPP5 are aligned with those of the other PP5 phosphatases (same as in Fig. 1) to illustrate their conserved sequence features. The structurally important hydrophobic ('b') and hydrophilic residues ('l'), and the two helices (A, B) are marked [8,9]. Residues numbered 4, 8, 11, 20, 24, and 27 are located on the same face of their respective helices, and may be involved in the recruitment of TPR-interacting proteins [8,9].

~69 kDa band in parallel Western analyses of the chromatographic fractions. In SDS-PAGE, the partially purified fraction contained a major polypeptide of the

expected size (69 kDa) (Fig. 4, lane 2). In Western blot, the rabbit antibody raised against the purified recombinant protein also specifically reacted with the native

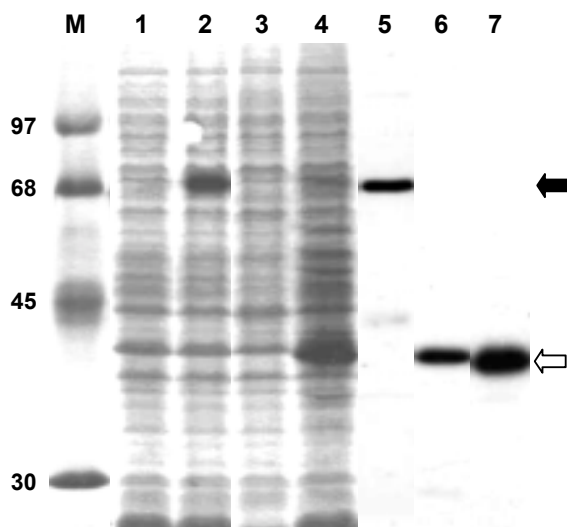


Figure 3

Bacterial expression of recombinant PfPPP5. Soluble S100 extract (approximately 30 μ g protein) of IPTG-induced (lanes 2, 4) or uninduced (lanes 1, 3) *E. coli* BL21(DE3)-RIG containing pET-15b-PfPPP5 (lanes 1, 2) or pET-15b- Δ TPR-PfPPP5 (lanes 3, 4) were analyzed by SDS-PAGE followed by staining of the gel with Coomassie Brilliant Blue R250 [12]. The respective purified proteins were analyzed in lanes 5, 6. Lane 7 represents about 5 μ g of purified His-tagged PfPPP5 digested with 50 ng of trypsin for 30 min at 30 $^{\circ}$. Protein markers (lane M) are indicated by their Mr in thousands.

polypeptide (Fig. 4, lanes 5, 6). A similar fraction prepared from uninfected erythrocytes contained very little protein (Fig 4, lane 3), and did not react with the antibody (lane 7). A pre-immune serum served as a negative control (Fig 4, lane 9). As expected, the native Pf protein was about 2 kDa smaller than the His-tagged recombinant (compare the native band in lanes 2, 5, and 6 with the recombinant in lanes 4 and 8). Using phosphohistone as substrate, the native Pf enzyme exhibited a specific activity of 210 ± 20 nmoles of 32 P per minute per mg enzyme, which is comparable to that of the recombinant enzyme. Together, these results demonstrated the equivalence of the native and recombinant proteins, and in turn, confirmed the authenticity of the cDNA sequence.

Primary structure of PfPPP5

When the predicted primary structure of PfPPP5 was aligned with known PP5 sequences (Fig. 1), the following features were obvious. First, PfPPP5 contained the catalytic core found in all Ser/Thr phosphatases of the PPP family, including the signature motifs such as GDXHGQ, GDFVDRG, RGNHE, HGLL, and SAPNYCD, to name a few [22,23]. Site-directed mutagenesis and structural

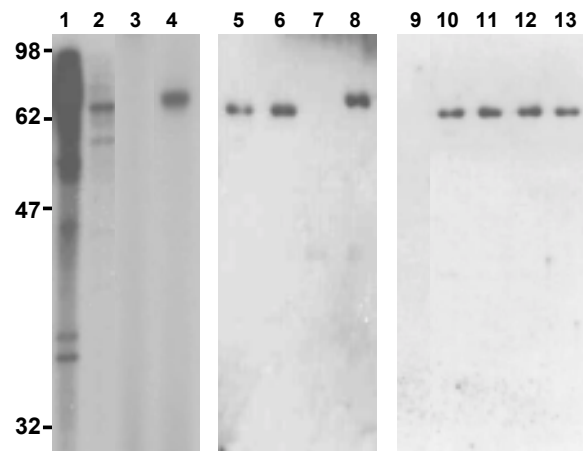


Figure 4

Analysis of native parasitic PP5. The following protein fractions from Pf were analyzed by SDS-PAGE followed by staining (lanes 1–4) or Western blot using a non-immune antibody (lane 9) or anti-PP5 antibody (lanes 5–8; 10–13): (1) S100 extract of Pf cells; (2) partially purified native PfPPP5 protein; (3) corresponding protein fraction from uninfected RBC; (4) purified recombinant (His)₆PfPPP5; (5–8) same fractions as lanes 1–4, respectively; (9) same fraction as in lane 1; (10–13) about 30 μ g total protein from asynchronous, late trophozoite, late schizont / segmented, and ring stage Pf, respectively. The Mr of protein markers are shown on left in units of thousands.

studies in PP1 and PP λ have previously established the roles of specific amino acid residues in these domains in the various aspects of catalysis, such as metal ion binding, phosphate recognition, and co-ordination of water molecules [24–26].

While the catalytic core is generally necessary and sufficient for the phosphohydrolase activity of PPP enzymes, the residues outside the core play critical roles in binding accessory proteins or small molecules that modulate the catalytic activity [25–29]. Specifically in the PP5 class, the N-terminus has been shown to contain three TPR motifs [5–8], the three-dimensional structure of which is now also available [8]. Interestingly, the N-terminal sequence of PfPPP5 was the longest of all, and seemed to contain four TPR motifs instead of three (Fig. 2). Thus, in comparing the full sequences of the PP5s, only three of the four PfPPP5 TPRs were aligned with the others, and the second TPR of PfPPP5 was left out. A detailed sequence analysis of all the PfPPP5 TPRs is offered below.

The TPR motif is a degenerate, 34-amino acid repeat that is often found in tandem arrays, sometimes separated by

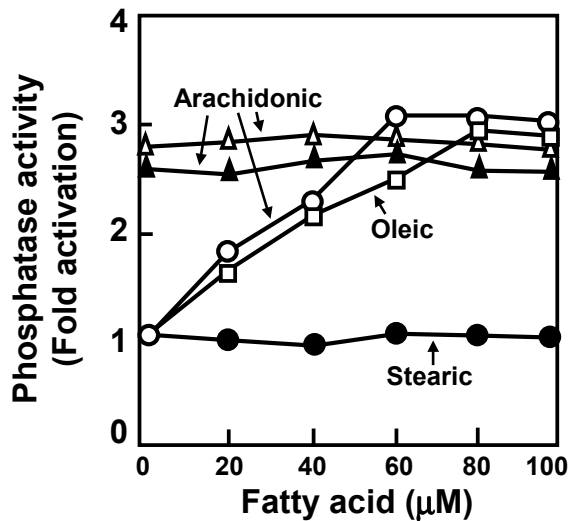


Figure 5

TPR-dependent activation of PfPP5 by unsaturated fatty acids. Phosphatase assays were performed using ^{32}P -phosphohistone substrate as described under Materials and Methods. The activities were expressed as the ratio of that obtained in the absence of any fatty acid, taken as 1. The following PfPP5 preparations were tested against the indicated fatty acids: recombinant Δ TPR-PfPP5 (open triangle); trypsin-treated recombinant PfPP5 (corresponding to lane 7 in Fig. 3; filled triangle); and recombinant PfPP5 (all the others).

spacer sequences [9]. We propose such a 22-residue spacer between the first two TPRs of PfPP5 (Fig. 1). Although no single amino acid is absolutely invariant in all TPRs, they do contain a largely conserved pattern of amino acid similarity in terms of size, hydrophobicity, and spacing [8,9]. Eight amino acid residues are critically placed on the same face of their respective helices: 4, 8, and 11 on the first helix, and 20, 24, and 27 on the second [8,9]. As shown in Fig. 2, residues that are identical or have similar hydrophobicity are found in all four proposed TPRs of PfPP5 in the correct relative spacing. Moreover, secondary structure prediction suggested that each PfPP5 TPR also has the potential to form the two conserved helices (A and B), as marked in Fig. 2. Thus, all the TPRs of PfPP5 may satisfy the structural requirements of a TPR motif.

An approximately 34-residue stretch following the last TPR of PP5 (Fig. 1) shows weak similarity to a TPR motif. However, alpha-helix prediction and structure determination have shown that it is a single long helix that extends out of the TPR domain [8]. Thus, this region may

not represent a typical TPR and therefore, is simply marked as "helix" in Fig. 1.

Auto-inhibitory role of PfPP5 TPR: activation by polyunsaturated fatty acids

The biochemical properties further confirmed the PP5-like nature of the Pf enzyme. First of all, members of the PP5 class are distinguished by their profound stimulation by polyunsaturated fatty acids [19–21,30]. As shown in Fig. 5, the phosphatase activity of PfPP5 increased with increasing concentrations of arachidonic acid and oleic acid. At its highest, the fatty-acid stimulated activity was about 3 times the basal activity and was exhibited at approximately 70 and 80 μM of arachidonic and oleic acids, respectively. In contrast, stearic acid, a saturated fatty acid, did not appreciably stimulate PfPP5 activity.

To test the role of TPR region in this activation, a TPR deletion mutant of PP5 (Δ TPR-PfPP5) starting at the Met-273 was expressed with an N-terminal (His)₆ tag. The recombinant protein was expressed from pET-15b clone and purified by Ni-chelation chromatography (Fig. 3, lane 6). In SDS-PAGE analysis, Δ TPR-PfPP5 exhibited a Mr of approximately 40 k as expected (predicted size of 37,098 for the PP5 part plus about 2,000 for the His₆ tag). Interestingly, the specific activity of the Δ TPR-PfPP5 enzyme was about 3-times that of the full-length PfPP5 without arachidonic acid (580 ± 35 nmoles of ^{32}P per minute per mg enzyme; Fig. 5). Moreover, unlike the full-length enzyme, Δ TPR-PfPP5 was not activated by arachidonic acid, suggesting that the TPR region is required for the activation.

These results are reminiscent of similar studies done in other PP5 enzymes in which mutational inactivation of the TPR domain resulted in an elevated basal activity and concomitant reduced response to unsaturated fatty acids [19]. Such studies led to the proposal that TPR domains regulate the PP5 catalytic region in a negative manner and that the interaction of arachidonic acid with TPR relieves this auto-inhibition. This was further supported by the demonstration that trypsin cleaved PP5 in the "hinge" sequence connecting the TPR region and catalytic domain, and that such cleavage produced a highly active enzyme which was refractory to further activation by fatty acids [18,20,21]. To test whether this is also true of PfPP5, we digested recombinant PfPP5 with trypsin, which resulted in the production of a trypsin-resistant fragment that was very similar in size to Δ TPR-PfPP5 (Fig. 3, lane 7). When assayed for phosphatase activity *in vitro*, the trypsin-digested PfPP5 indeed behaved like Δ TPR-PfPP5 in that it was about 3-fold more active than the full-length enzyme and was not activated by arachidonic acid any further (Fig. 5). Together these results

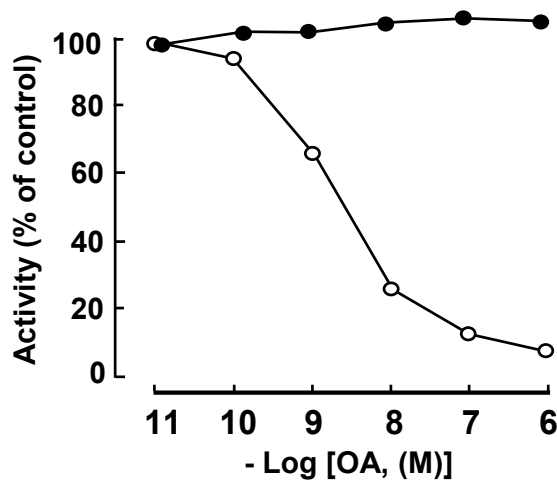


Figure 6

Okadaic acid sensitivity of PfPP5. Phosphatase assay using recombinant PfPP5 (open circles) and the control OA-resistant recombinant PfPP (closed circles) were carried out at various concentrations of OA using ^{32}P -phosphohistone substrate as described under Materials and Methods. Values of a control reaction lacking OA were taken as 100.

suggest a role of PfPP5 TPR in auto-inhibition and in fatty acid-mediated activation.

Okadaic acid sensitivity of PfPP5

The differential sensitivity of the members of the PPP family to biological toxins has been exploited as a diagnostic tool in studying these phosphatases. The marine toxin okadaic acid (OA), in particular, inhibits the PP2A class of phosphatases at sub-nanomolar concentrations ($\text{IC}_{50} \sim 0.05 \text{ nM}$) and the PP1 class in the low nanomolar range ($\text{IC}_{50} \sim 5\text{--}45 \text{ nM}$). The SAPNYC motif of PPP enzymes is essential for OA-binding, and is found in all OA-sensitive phosphatases including PP1, PP2A, and PP5 [[25,27,28], and references therein]. This motif is also present in PfPP5 (Fig. 1). Phosphatases lacking this motif include the PP2B and PP2C classes, which are thus highly resistant to OA. PP2A contains an additional Cys residue two amino acids downstream (underlined in SAPNYCYRCG), believed to be important for the profound sensitivity of this class to OA [28], and this Cys is absent in PfPP5 and other PP5 enzymes (Fig. 1). As shown in Fig. 6, OA inhibited PfPP5 in a dose-dependent manner, with an IC_{50} of $5.1 \pm 0.2 \text{ nM}$. This moderate OA-sensitivity further establishes the identity of PfPP5, since other PP5 enzymes also exhibit an IC_{50} value for OA in the 4–6 nM range [18]. The ΔTPR mutant exhibited an essentially identical IC_{50} (not shown), supporting the

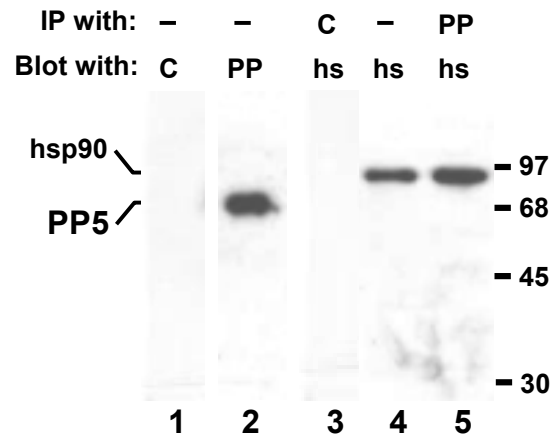


Figure 7

Complex formation between PfPP5 and Pfhsp90. The following abbreviations have been used for the antibodies (labeled on top): C = non-immune serum; PP = anti-PP5; hs = anti-hsp90. The protein bands (Western blot) are indicated by hsp90 or PP5 (on the left), and the sizes of the protein standards are indicated (on the right) by their Mr values in thousands. Lane numbers are indicated on the bottom. About 0.5 mg of Pf cell pellet was lysed and subjected to immunoprecipitation (IP) with either a rabbit antibody against PP5 (lane 5) or a control non-immune serum (lanes 3) as described under Materials and Methods. The precipitate was analyzed in SDS-PAGE followed by Western blot using anti-PP5 (lane 2), anti-hsp90 (lanes 3–5), or non-immune serum. Total Pf cell extract (40 μg) was also directly subjected to SDS-PAGE and Western blot without immunoprecipitation (lanes 1, 2, 4).

notion that the TPR domains do not play a direct role in the interaction with OA.

PfPP5 binds Pf heat shock protein 90 (hsp90)

Hsp90 is a highly conserved molecular chaperone necessary for viability and for proper folding, processing, and function of proteins involved in several signal transduction pathways [31,32]. Interestingly, mammalian hsp90 has been shown to specifically interact with PP5, and this interaction may have important consequences in PP5 structure and function [33,34]. An hsp90 ortholog has been described in Pf [35], but its function is currently unknown. It was, therefore, logical for us to ask whether an interaction between PP5 and hsp90 also occurs in Pf. To initiate these studies, we performed a co-immunoprecipitation analysis of Pf extracts in which complexes containing PP5 were first precipitated with anti-PP5 antibody and the precipitate then probed with anti-hsp90 antibody in Western blot. Results (Fig. 7) show that Pfhsp90 is indeed found in complex with PfPP5, suggesting a potential role of this interaction in the mu-

tual regulation of these two important proteins. The "control" non-immune serum did not react with either protein (lane 1) and did not precipitate hsp90 (lane 3).

Discussion

We provide biochemical and molecular evidence for a PP5 protein Ser/Thr phosphatase of *P. falciparum* that has the catalytic core of the PPP superfamily and four tetratricopeptide (34-residue) repeat (TPR) domains (Fig. 1, 2). We have therefore, tentatively named this enzyme PfPP5. To our knowledge, PfPP5 is the only TPR-containing *Plasmodium* protein reported at this time. These domains, and the associated properties such as activation by unsaturated fatty acids are by far the most noteworthy features of PfPP5.

Recently, a PP5 phosphatase was identified in *Trypanosoma* [30] that contained the prototype PP5 primary structure, including three TPR domains at the N-terminus. While mammalian PP5 is activated 10–40 fold by arachidonic acid, the *Trypanosoma* PP5, like its *Plasmodium* counterpart (Fig. 5), was also activated only modestly, about 3-fold. Although the reason behind the lower activation potential is unknown, at least in PfPP5 it correlates with a similar fold activation following loss of the TPR domains through either mutagenesis or proteolytic cleavage (Fig. 3, 5). Thus, it appears that the TPR domains of the primitive parasitic PP5 may be less auto-inhibitory than those of their higher eukaryotic counterparts. Recent mutational analysis of recombinant PP5 has identified sequences important in autoinhibition [19]. Mutation of Glu-76 (underlined in the human PP5 in Fig. 1) whose side chain projects away from the TPR groove, resulted in a 10-fold elevation of basal activity, and this Glu is conserved in both Pf and *Trypanosoma* PP5 (Fig. 1). A role of the C-terminal end in autoinhibition was conjectured based on the finding that mutation of Gln-495 (underlined in Fig. 1) elevated basal activity by 10-fold [19]. This residue is also found in Pf (Gln-592) although it is not conserved in a number of other PP5 sequences (Fig. 1). Clearly, detailed mutational analysis of these domains should shed light on the mechanism of PfPP5 autoinhibition and the role of polyunsaturated fatty acids in preventing it.

The TPR motif was originally identified as a protein interaction module in the cell division cycle (CDC) proteins in yeast [36,37] but has since been shown to occur in proteins with diverse functions in a variety of species [9]. The number of the TPR domains, the spacing between them, and their placement in the primary structure of the protein appear variable, and the relative contribution of the individual TPRs in a given protein has not been studied. At least four major kinds of macromolecular complexes have been identified that involve TPR domains.

These include complexes involved in anaphase promotion, transcription repression, protein import, and molecular chaperoning [9,10]. Yeast two-hybrid analysis and *in vitro* interaction studies have shown that the TPR domain of PP5 interacts with the TPR domains of CDC16 and CDC27, which may be important in recruiting PP5 to the anaphase promoting complex (APC) and to the mitotic spindles during cell division (38). Interaction of PP5 with heat shock protein 90, a chaperone, has received special attention because of its potential to regulate a variety of pathways. For example, PP5 is a major component of mature glucocorticoid receptor complexes of higher eukaryotes and appears to regulate glucocorticoid receptor function *in vivo* [[34] and references therein]. Such observations suggest that PP5 may act as a co-chaperone for hsp90 and raise the possibility that protein dephosphorylation may play an important regulatory role in protein folding [33,34]. Interestingly, recent studies have identified a few residues of mammalian PP5 critically important for its interaction with hsp90 [34]. As marked on the human PP5 TPR (underlined in Fig. 1) the corresponding residues are: Lys-32, Lys-97, and Arg-101. It is notable that the corresponding residues are also conserved in PfPP5 TPR. The availability of full-length and TPR-deleted recombinant PfPP5 clones will now allow us to further investigate the exact nature of the PfPP5-Pfhsp90 complexes, and map the interacting PfPP5 residues through site-directed mutagenesis. Clearly, the discovery of PfPP5 and its interaction with homologous hsp90 will open up new directions in parasitic protein/ gene regulation and signaling, and may lead to specific drug targets.

Conclusions

PfPP5 represents the first TPR-containing protein discovered in the *Apicomplexan* parasites. In view of the essential role of TPR domains in protein-protein interaction, further studies of PfPP5 and its TPR domain will shed new light on phosphatase regulation and the role of PP5 in these clinically important parasites. As with mammalian PP5, PfPP5 may also play an important role in cell division through its interaction with parasitic CDC proteins. Interaction between the *Plasmodium* PP5 and heat shock protein 90 may reflect a mutual relationship between protein phosphorylation and protein folding in this parasite. The role of TPR in this interaction can be addressed through structure-function analysis of the recombinant proteins.

Materials and methods

PCR and other procedures

The following oligodeoxynucleotide primers were designed against the putative PfPP5 sequence found on chromosome 13 (ALO49185; *Plasmodium falciparum* chr13_002073): ATGTTACACAACCATGAT-

GTAGAAGAAG;TAAATGTTTTGATACAAATTAT-GAGC. These primers were used to amplify the PfPP5 gene or the cDNA using the Pf3D7 genomic DNA or Dd2 total RNA as template with PCR and RT-PCR, respectively. The RT reaction was performed using AMV reverse transcriptase, and all PCRs were performed with a 15:1 mixture of Taq:Pfu polymerase. The products were cloned into pGEM-T and confirmed by sequencing. The genomic and cDNA sequences were identical. This sequence was then subcloned into the XhoI and BamHI sites of the pET-15b vector by designing similar primers but containing the respective restriction sites. The TPR deletion mutant, starting at Glu-274 (bold in Fig. 1), was also amplified in a similar manner using specific primers, and then cloned. Introduction of the plasmids into *E. coli* BL21(DE3) cells containing the compatible RIG plasmid [39], induction of the proteins with IPTG, and purification through Ni-chelation chromatography were performed using the His-Bind kit reagents and procedure (Novagen).

Antibodies against PP5 and hsp90 were raised in rabbit and mouse, respectively, using standard procedures [40]. Fatty acids were purchased from Sigma (St. Louis, MO). SDS-PAGE and Western blot using chemiluminescence-based detection were performed essentially as described [15]. Immunoprecipitation of PfPP5 complexes followed by immunoblot analysis of the precipitate were carried out essentially as described [34].

Growth of *Plasmodium* and purification of PfPP5

P. falciparum (3D7 or Dd2) was grown in human A-positive erythrocytes and infected cells were harvested at about 15% parasitemia and lysed by the saponin method essentially as described [14,15]. PfPP5 was partially purified using published procedures [18]. In brief, the parasites were resuspended in buffer A (50 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM DTT) plus 20 mM NaCl, 1% Triton X-100, leupeptin (1 µg/ml), and aprotinin (1 µg/ml) at an approximate protein concentration of 10 mg per ml. The suspension was sonicated and the lysate centrifuged at 100,000 × g for 30 min. The supernatant, called "total extract", was subjected to 45%–60% ammonium sulfate fractionation. The pellet was resuspended in 10 ml of buffer B (20 mM HEPES, pH 8.5, 1 mM EDTA, 1 mM DTT), desalted on a Sephadex G-25 column equilibrated with buffer B, then subjected to sequential chromatography on a 5 ml HiTrap heparin column (Pharmacia) and then a 5 ml HiTrap Q column (Pharmacia). PP5 activity was followed during the purification by assaying trypsin-stimulated pNPPase activity. The fractions were further confirmed by Western blot using anti-PfPP5 antibody. Parasite culture was synchronized essentially as described earlier [15].

Phosphatase assay

Phosphatase activity against pNPP or phosphohistone substrates was determined essentially as described previously. Standard assays were performed in buffer A containing 1 mM EDTA, and additional activators or inhibitors were added only where mentioned. Reactions were incubated at 37°C. ³²P-labeled phosphohistone was made as described [14,15,18]. When using phosphohistone as substrate, reactions were carried out in 80 µl followed by quantification of the liberated ³²Pi by phosphomolybdate extraction assay [14,15,18]. Reactions were followed with time, and results were corrected by subtraction of the corresponding values from an enzyme-free reaction. When *p*-nitrophenylphosphate was used as substrate (at 10 mM), the reaction was scaled up to 200 µl, and the liberated *p*-nitrophenol was quantitated by measurement of absorbance at 415 nm [14,23]. Phosphatase reactions were routinely initiated with the addition of substrate. Dephosphorylation was kept to less than 10 % of the total phosphorylated substrate, and the reaction was linear with respect to enzyme concentration and time. When using potential activators (e.g., fatty acids) or inhibitors (e.g., okadaic acid), they were added to the enzyme mixture 10 minutes prior to initiating the reaction with the addition of substrate [14].

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