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RESEARCH ARTICLE

Characterization of *Plasmodium falciparum* co-chaperone p23: its intrinsic chaperone activity and interaction with Hsp90

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Abstract It is well known that the co-chaperone p23 regulates Hsp90 chaperone activity in protein folding. In *Plasmodium falciparum*, a putative p23 (Pfp23) has been identified through genome analysis, but its authenticity has remained unconfirmed since co-immunoprecipitation experiments failed to show its interaction with *P. falciparum* Hsp90 (PfHsp90). Thus, recombinant Pfp23 and PfHsp90 proteins purified from expressed clones were used in this study. It was clear that Pfp23 exhibited chaperone activity by virtue of its ability to suppress citrate synthase aggregation at 45°C. Pfp23 was also shown to interact with PfHsp90 and to suppress its ATPase activity. Analyses of modeled Pfp23-PfHsp90 protein complex and site-directed mutagenesis further revealed strategically placed amino acid residues, K91, H93, W94 and K96, in Pfp23 to be crucial for binding PfHsp90. Collectively, this study has provided experimental evidence for the inherent chaperone function of Pfp23 and its interaction with PfHsp90, a sequel widely required for client protein activation.

Keywords Heat shock protein 90 · p23 · *Plasmodium falciparum* · Chaperone · Site-directed mutagenesis

Introduction

The co-chaperone, p23, is a small protein involved in the cascade of Hsp90 chaperone activity [1, 2]. It is well documented that Hsp90 folds and activates proteins essential in cellular signaling and development (e.g., kinases and transcription factors) by undergoing a cycle of conformational changes driven by the binding and hydrolysis of ATP at its N-terminal domain [3–6]. The interaction between p23 and Hsp90 occurs at the late stage of the chaperone cycle and is ATP-dependent [7–9]. Reportedly, the binding of p23 reduces the rate of ATP hydrolysis and traps Hsp90 in the ATP-bound conformation [10]. It was also proposed that p23 binding prolongs the interaction between Hsp90 and its client proteins, thereby securing their maturation. Subsequently, the hydrolysis of ATP leads to the release of activated client proteins [11]. This latter feature was demonstrated using an in vitro reconstituted Hsp90 chaperone system, whereby the incorporation of p23 significantly increased the competence of mature progesterone and glucocorticoid receptors in binding steroid hormones [12, 13], as such attesting to p23's importance in driving the Hsp90 functional repertoire.

In the recently resolved crystal structure complex of Hsp82 and SbaI, homologues of Hsp90 and p23 in yeast, respectively, the two proteins were shown to interact through their N-terminal domains [14]. Hsp82 was crystallized in the ATP-bound conformation and possessed a closed 'lid' segment positioned over the ATP-binding pocket, which would otherwise remain open in the absence of ATP. In the case of SbaI, the N-terminal domain was found to bind over the ATP-binding pocket of Hsp82, with the closed 'lid' forming one of the interaction interfaces. The requirement for this ATP-induced closed 'lid' conformation in Hsp82 to interact with SbaI is circumstantially supported by experiments showing the preferential binding of p23 to ATP-bound Hsp90 [7–9].

Apart from regulating Hsp90 chaperone functions, p23 is also capable of binding partially folded proteins and

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suppressing protein aggregation in vitro [15, 16]. This has led to the suggestion that p23 may itself function as a chaperone, independent of Hsp90. When the C-terminal tail of p23 was truncated, its chaperone activity was abolished, suggesting that the acidic amino acid-rich tail may be instrumental for its function [17]. This intrinsic chaperone activity of p23 has been demonstrated to be essential for the recycling of steroid receptors and telomerase [18, 19].

In *Plasmodium falciparum*, the species that causes the most severe form of human malaria, a putative homologue of p23 (Pfp23) has been reported [20]. Pfp23 was initially identified as a 36-kDa major phosphoprotein associated with the kinase activity of an unknown protein in *P. falciparum* [21, 22]. Subsequent procurement of its protein sequence revealed its homology to the co-chaperone p23 [20]. However, validation of its role as a co-chaperone of *P. falciparum* Hsp90 (PfHsp90) has been unsuccessful so far [20, 23]. Co-immunoprecipitation experiments performed using either antibodies specific to Pfp23 or PfHsp90 did not detect complexes containing both the proteins. However, as alluded to earlier, p23 has important roles in the activation of a plethora of Hsp90 client proteins, many of which are crucial in signal transduction and regulating cell growth. Thus, it would be unusual to exclude its role in *P. falciparum*, as implicated in reports so far. Therefore, in this study, the putative Pfp23 was probed for its intrinsic chaperone activity and interaction with PfHsp90.

Materials and methods

Cloning of Pfp23 and PfHsp90

The DNA sequences of Pfp23 (PF14_0510) and PfHsp90 (PF07_0029) were retrieved from PlasmoDB, and primers were designed to clone these genes (Table 1). The genes were cloned from the mRNA of *P. falciparum* 3D7. The mRNA extraction and reverse transcription procedures were carried out as previously described [24].

Briefly, the PCR-amplified gene products were separated by agarose gel electrophoresis using 0.8% (*w*/*v*) agarose gel, and the bands that corresponded to the expected size of the genes were excised and purified using the MinElute™ Gel Extraction Kit (Qiagen). Subsequently, the purified fragments were ligated into pCR-BluntII-TOPO Vector using the Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen). The plasmids of the recombinant clones were extracted using the Wizard® *Plus* SV Minipreps DNA Purification System from Promega. Full sequencing was carried out using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer and analyzed using the ABI PRISM 3100 DNA sequencer to identify recombinant clones that contained the genes of interest.

Subsequently, the recombinant clones that contained *Pfp23* and *PfHsp90* were double-digested with *Bam*HI/*Sal*I and *Bam*HI/*Xho*I, respectively. *Pfp23* was subcloned into the expression vector, pET24a (Novagen), whereas *PfHsp90* was ligated to pET24a and pGK [25] expression vectors. PfHsp90 expressed using the pGK vector contained a GST- and a His_6 tag and would be used for the GST pull-down assay. However, the more highly expressed PfHsp90 from the recombinant pET24a vector possessed only a His_6 tag. This fusion protein would be used for the ATPase assay. The ligated recombinant vectors were transformed into *Escherichia coli* BL21 (DE3) cells that contained a RIG plasmid [26] for expression of the recombinant fusion proteins. DNA sequencing was performed to authenticate the recombinant clones carrying the genes of interest.

Construction of Pfp23-truncated and single mutants

The recombinant pET24a plasmid that contained *Pfp23* was used as a template for the construction of Pfp23-truncated mutants. Pfp23-truncated mutants T1, T2, T3, T4 and T5 were PCR-amplified using appropriate forward primers and a common reverse primer used for Pfp23 wild-type (Pfp23-WT) previously. On the other hand, amplifications of Pfp23-truncated mutants, Pfp23-(Δ135–275) and Pfp23-(Δ220–275) were carried out using appropriate designed reverse primers and a common forward primer from Pfp23-WT (Table 1). Similar cloning procedures described earlier were adopted for the subsequent steps.

The QuikChange™ Site-directed Mutagenesis Kit from Stratagene was used for the construction of Pfp23 single mutants [27]. Primers were designed to introduce the desired mutations using PCR protocols described previously [28]. DNA sequencing was performed subsequently to verify the changes in the nucleotide sequences prior to transformation into *E. coli* BL21 (DE3) with the RIG plasmid for recombinant fusion protein expression.

Expression and purification of Pfp23 and PfHsp90

Bacterial cultures carrying the PfHsp90 and Pfp23 recombinant plasmids were grown in 100 ml Luria-Bertani broth with 50 μg/ml kanamycin (Sigma) and 40 μg/ml chloramphenicol (Sigma) for protein expression. The cultures were grown at 37°C with agitation at 250 rpm until an OD_{600} of 0.8 was reached. Protein expression was induced by the addition of 1 mM isopropyl-*β*-D-1-thiogalactopyranoside (Sigma) followed by incubation at 16°C with shaking at 250 rpm for 16 h.

Table 1 Primers designed for gene cloning and mutations

Gene constructs	Primers[a] (5′ to 3′)
PfHsp90	F: ATCATTggatccATGTCAACGGAAACATTCGC
	R: CTGGATctcgagGTCAACTTCTTCCATTTTAGAATCGG
Pfp23	F: ATCGTAggatccATGCCACTCTATCC
	R: ATGAATgtcgacGGCTACTGGTTCTTGTACTTCTACTGC
Pfp23-truncated mutants:	
T1	F: ggatccATGATAGAAAATGTAAAGATTGACC
T2	F: ggatccATGATAGAAAATGTAAAGATTGACC
T3	F: ggatccATGATAATAAAAAAAGAACAAGAAAGATGG
T4	F: ggatccATGAACTCCTGGGTAGATACCGATG
T5	F: ggatccATGGGAGGTATGCCAGATATGAGTC
Pfp23-(Δ135–275)	R: gtcgacAAATTGACTCATATCTGGCATACC
Pfp23-(Δ220–275)	R: gtcgacATTCATATTATTCATATTAGGC
Pfp23 single mutants[b]*:*	
K91A	F: ATAACGATGGA**GCG**AAACACTGGGTTAAATG
	R: ACCCAGTGTTT**CGC**TCCATCGTTATTTAAAG
K92A	F: CGATGGAAAG**GCA**CACTGGGTTAAATG
	R: CATTTAACCCAGTG**TGC**CTTTCCATCG
H93S	F: ATGGAAAGAAA**AGC**TGGGTTAAATGTGAC
	R: CACATTTAACCCA**GCT**TTTCTTTCCATCG
W94S	F: GGAAAGAAACAC**AGC**GTTAAATGTGACTG
	R: GTCACATTTAAC**GCT**GTGTTTCTTTCCATCG
V95N	F: GAAACACTGG**AAT**AAATGTGAC
	R: GTCACATTT**ATT**CCAGTGTTTC
K96A	F: AAACACTGGGTT**GCA**TGTGACTGGAACTCC
	R: AGTTCCAGTCACA**TGC**AACCCAGTGTTTC

[a] Restriction enzyme sites are in lower case, *F* represents forward primer, *R* represents reverse primer

[b] Codons mutated in Pfp23 are *bold* and *underlined*

Following induction, the cultures were harvested by centrifugation at 6,000 rpm for 5 min at 4°C. The supernatant was discarded, and the pelleted cells were resuspended in cold 50 mM Tris-HCl, pH 7.5 buffer. The cells were lysed via sonication with a 1/8-inch probe at an intensity of 10 μm for eight cycles with 10-s pulse and 10-s rest in each cycle. Cell lysates were subsequently centrifuged at 12,000 rpm for 20 min, and the soluble protein fractions that contained the fusion proteins of interest were subjected to purification.

The PfHsp90 and Pfp23 recombinant proteins, both carrying a C-terminal His_6 tag, were purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen). For each purification, an aliquot of 1 ml of the soluble protein fraction was incubated with 50 μl of Ni-NTA beads for 15 min at 4°C. Unbound proteins were removed by washing thrice with 250 μl of 50 mM Tris-HCl, pH 7.5, 20 mM imidazole buffer. The recombinant proteins were eluted in 50 μl of 50 mM Tris-HCl, pH 7.5, 300 mM imidazole buffer.

To remove co-purified contaminating proteins, the eluted proteins were further purified by gel filtration chromatography on a Superdex 200 column (GE Healthcare). The recombinant proteins were separated at a flow rate of 0.5 ml/min in buffer containing 50 mM Tris-HCl, pH 7.5. The eluted samples were analyzed by SDS-PAGE to determine the fractions that contained the recombinant proteins of interest. These fractions were pooled and concentrated using Amicon Ultra-4 Centrifugal Filter Units with appropriate molecular weight cutoff and stored at 4°C for subsequent assays. The concentrations of the purified fusion proteins were determined by Bradford assay (Biorad) using bovine serum albumin (Sigma) as a standard. Expression and purification of the recombinant proteins were upscaled proportionately when necessary to obtain a sufficient amount of recombinant proteins for the subsequent assays.

Citrate synthase aggregation assay

The intrinsic Hsp90-independent chaperone activity of Pfp23 was assayed based on the aggregation of citrate synthase [29] using a slightly modified protocol. Porcine citrate synthase (Sigma) was dialyzed with 40 mM HEPES, pH 7.5 buffer, overnight and concentrated to an 8.5-μM stock solution. Aggregation was induced by the

incubation of citrate synthase (0.15 μM) in 40 mM HEPES, pH 7.5 buffer, at 45°C. The assays were performed in the presence or absence of five-fold excess (0.75 μM) of Pfp23-WT and Pfp23-(Δ220–275). The extent of aggregation was measured by the increase in absorbance due to light scattering and monitored at 334 nm using the Tecan Safire spectrophotometer. The absorbance was recorded every 2.5 min for 20 min.

In vitro interaction between Pfp23 and PfHsp90

The in vitro interaction of Pfp23 and PfHsp90 was investigated using GST pull-down assay. Nickel affinity purified dual-tagged PfHsp90 fusion protein expressed from the pGK vector was used as a bait. For each experiment, 2 μg of PfHsp90 fusion protein was incubated with 50 μl of glutathione Sepharose beads (GE Healthcare) at 4°C for 15 min. The bound fusion proteins were washed thrice with 50 mM of Tris-HCl, pH 7.5, buffer to remove unbound proteins. Subsequently, the beads were reconstituted in 500 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM dithiothreitol, 0.01% (*v*/*v*) Triton X-100). The binding assays were carried out in the presence of 5 mM $MgCl_2$, 5 mM ATP and 10 μg of Pfp23, unless stated otherwise. Samples were incubated at 30°C for 30 min for PfHsp90-Pfp23 complex formation. Thereafter, the samples were washed thrice with 500 μl of binding buffer prior to elution in 150 μl of reduced glutathione buffer. Fifty microliters of the eluted sample was analyzed by SDS-PAGE. Gel image analyses and densitometric measurements were performed using Bio-Rad Molecular Imager Gel Doc XR system equipped with the Quantity One® Software.

ATPase assay

The ATPase activity of PfHsp90 was measured using the pyruvate kinase/lactate dehydrogenase-coupled assay [6, 30]. The assay was carried out in a 200-μl reaction containing 2 μM PfHsp90 incubated in 100 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 20 mM KCl, 0.8 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 50 μg/ml lactate dehydrogenase and 0.2 mg/ml pyruvate kinase at 37°C. The decrease in NADH level, which is in direct stoichiometry to the amount of ATP hydrolysed, was determined spectrophotometrically at 340 nm. The authenticity of PfHsp90 ATPase activity was verified by its sensitivity to inhibition by radicicol (100 μM), a specific Hsp90 ATPase inhibitor. To determine the effect of Pfp23 interaction on PfHsp90 ATPase activity, Pfp23 was added to the assay reactions in increasing concentrations (2–30 μM). In addition, Pfp23 single mutants with differential binding capacity to PfHsp90 were also tested for their influence on PfHsp90 ATPase activity. The Pfp23 single mutants were added in ten-fold molar excess (20 μM), and their effects on the ATPase activity of PfHsp90 were compared to that of Pfp23-WT. All measurements were made using the Tecan Safire spectrophotometer. The ATPase activity reported is expressed as micromole of ATP hydrolysed per micromole of PfHsp90 in 1 min at 37°C.

Bio-computational analyses of Pfp23 and PfHsp90

Protein sequence alignment was performed using the CLUSTAL W Multiple Alignment Program [31], and the percentage identities were determined by MatGAT 2.02 [32]. Tertiary structures of Pfp23 and PfHsp90 were modeled by the SWISS-MODEL program [33] using the "First Approach Mode". The Hsp82-SbaI crystal structure complex (PDB accession number: 2cg9) was used as a template for protein modeling. The modeled protein structures were subsequently visualized using the Swiss-PdbViewer (SPdbV) program [34].

Results

Bio-computational analyses of the putative Pfp23

P. falciparum 3D7 genome encodes a putative p23 homologue of 275 amino acid residues. The Pfp23 possesses the characteristic "Hsp20-like chaperone superfamily" domain (residues 2-106) and a C-terminal tail rich in acidic residues. The "Hsp20-like chaperone superfamily" domain, which is also present in the yeast SbaI, is known to be important for Hsp82 interaction [14]. Sequence homology analyses indicated that Pfp23 shared 44% sequence identity with *Schizosaccharomyces pombe* p23 homologue [20], but exhibited only 19.6 and 23.6% sequence identities in common with the human p23 and *Saccharomyces cerevisiae* SbaI, respectively. In addition, the putative Pfp23 (275 residues) has a longer protein sequence than the human p23 (160 residues) and *S. cerevisiae* SbaI (216 residues). Multiple sequence alignment further revealed the difference in protein length of Pfp23 lies in the presence of the unique tandem repeats, **GNMGGL**x7 (between amino acid residues 135–176) and a longer C-terminal tail (Fig. 1). Similar glycine-rich tandem repeats were also found in p23 of other *Plasmodium* species, namely *P. chabaudi*, *P. berghei*, *P. yeolii* and *P. knowlesi* [20]. In comparison, the human p23 only possessed a *GGD* sequence, whereas the yeast SbaI has two repeats of *GGAGGA* and a single *GGAGGM* sequence. So far, the significance of these glycine-rich sequences has yet to be reported and as such its functionality remains enigmatic.

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S.cerevisiae      MSDKVINPQVAWAQRSSTTDPERNYVLITVSIADCDAPELTIKPSYIELKAQSKPHVGDE 60
H.sapiens         ----MQPASAKWYDR-------RDYVFIEFCVEDSKDVNVNFEKSKLTFSCLG----GSD 45
P.falciparum      ---MPLYPIVLWAQK-------KECLYLTIELQDIENVKIDLKEDKLYF-------YGTK 43
                         . . * ::       :: : : . : * .  :: :: . : :        * .

S.cerevisiae      NVHHYQLHIDLYKEIIPEKTMHKVANGQHYFLKLYKKDLESEYWPRLTKEKVKYPYIKTD 120
H.sapiens         NFKHLN-EIDLFHCIDPNDSKHKRTD--RSILCCLRKGESGQSWPRLTKERAKLNWLSVD 102
P.falciparum      DKNEYEFTLNFLKPINVEESKYSTQR--NIKFKIIKK--EQERWKTLNND-GKKHWVKCD 98
                  : :. :  ::: : *  :.: :.     .  :   :*  . : *  *.::  *  ::. *

S.cerevisiae      FDKWVDEDEQDEV-------------------------------------------EA 135
H.sapiens         FNNWKDWEDDSDE-------------------------------------------DM 117
P.falciparum      WNSWVDTDEEDKANDYDDMGMNSFGGMGGMPDMSQFGNMGGLGNMGGLGNMGGLGNMGGL 158
                  ::.* * :::..

S.cerevisiae      EGNDAAQGMDFSQMMGGAGGAGGAGGMDFSQMMGG-------AGGAGSPDMAQLQQLLAQ 188
H.sapiens         SNFDR-----FSEMMN---NMGGDEDVDLPEVDG--------------------------- 143
P.falciparum      GNMGGLGNMGGLGNMGGLGNMGGMGDLDFSKLGNMGGDMPNFAGLGGMDQFKNMPNMNNM 218
                   . .          *.   . **  .:*:.:: .

S.cerevisiae      SGGNLDMGDFKENDEEDEEEEIEPEVKA----------------------------- 216
H.sapiens         ----------ADDDSQDSDDEKMPDLE----------------------------- 160
P.falciparum      NDDDSSSYGDDTSDEEDDDDEEDEDVEVDNKTLDSDKLKDEENKIPDAAVEVQEPVA 275
                            .*.:*.::*   :::
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Fig. 1 Protein sequence alignment of p23 and related sequences. Protein sequences of *Homo sapiens* p23 (NP_006592.3) and *Saccharomyces cerevisiae* SbaI (NP_012805.1) were obtained from GenBank, whereas that of Pfp23 (PF14_0510) was retrieved from PlasmoDB. The *P. falciparum* genome encodes a longer putative p23 homologue that shares only 19.6 and 23.6% amino acid sequence identities with the human p23 and *S. cerevisiae* SbaI, respectively. The glycine-rich **GNMGGL**x7 sequence repeats in Pfp23 are *bold* and *underlined*. *Asterisk* identical amino acid residues in all the sequences, *colon* indicates conserved amino acid substitutions, *dot* indicates semi-conserved amino acid substitutions

Cloning, expression and purification of Pfp23 and PfHsp90

To clone the putative Pfp23 and PfHsp90, reverse transcription and PCR amplifications were carried out using appropriate primers and mRNA from *P. falciparum* 3D7. The resultant ORF of *Pfp23* was inserted into the pET24a expression vector, whereas that of *PfHsp90* was ligated into pET24a and pGK expression vectors. Subsequently, DNA sequencing was performed to verify their authenticities. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) already harboring the RIG plasmid. The RIG plasmid encodes tRNAs that recognize rare codons present in the AT-rich plasmodial genes [26]. Hence, it was included in anticipation of improving the expression of the recombinant proteins.

Pfp23 and PfHsp90 were solubly expressed as recombinant proteins. The PfHsp90 fusion protein expressed from the pGK vector contained an N-terminal GST tag and a C-terminal His_6 tag. The inclusion of a dual tag allowed an initial purification of PfHsp90 fusion protein using Ni^{2+} affinity chromatography and gel filtration separation, prior to immobilization on the glutathione Sepharose for the GST pull-down assays. As the subsequent ATPase assay requires large quantities of highly purified proteins, the recombinant PfHsp90 was expressed using the pET24a vector. The recombinant pET24a vector provided significantly higher quantities of PfHsp90 fusion proteins compared to the recombinant pGK vector. Pfp23 was also recombinantly expressed using the pET24a vector, and similar Ni^{2+} affinity chromatography and gel filtration separation techniques were utilized to purify these fusion proteins. After electrophoretic separation, the recombinant Pfp23 was found to be approximately 36 kDa, whereas PfHsp90 expressed from recombinant pGK and pET24a vectors were 114 kDa and 88 kDa, respectively (Fig. 2).

The recombinant Pfp23 possesses chaperone activity

Prior to this study, the functionality of the putative Pfp23 had not been demonstrated. In view of this, Pfp23 was examined for its intrinsic chaperone activity in preventing the aggregation of citrate synthase [29]. Pfp23 was added to citrate synthase in a 5:1 M ratio and incubated at 45°C. Aggregation of citrate synthase was detected by the increase in absorbance at 334 nm over a 20-min interaction time.

In the absence of Pfp23, citrate synthase aggregated spontaneously at 45°C (Fig. 3). The addition of Pfp23 suppressed the aggregation of citrate synthase, as reflected by the decrease in absorbance reading. Similar anti-aggregation activity was observed in human p23, but was abolished when its acidic C-terminal tail was truncated by 30 amino acid residues [17]. To investigate whether the C-terminal tail of Pfp23 also contributes to anti-aggregation activity, a mutant clone of Pfp23 that lacks the C-terminal acidic residues was constructed. Deletion of the C-terminal tail in Pfp23-(Δ220–275) resulted in a total loss of the anti-aggregation activity observed in Pfp23-WT, implying the requirement of the C-terminal tail for its full chaperone activity. No self-aggregation of Pfp23-WT and Pfp23-(Δ220–275) was observed at 45°C in the control

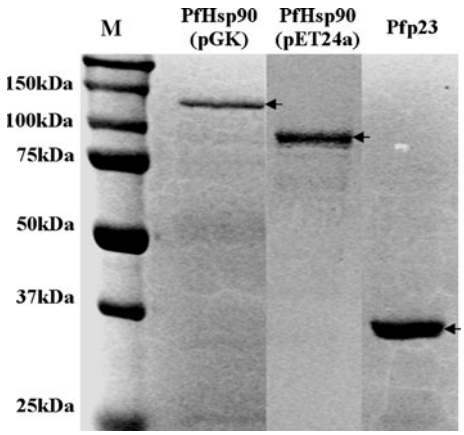

Fig. 2 SDS-PAGE analyses of purified Pfp23 and PfHsp90 recombinant proteins. PfHsp90 was expressed from recombinant pGK and pET24a vectors separately. PfHsp90 expressed from the pGK vector contained an N-terminal GST tag and a C-terminal His_6 tag, whereas PfHsp90 from the pET24a vector possessed only the C-terminal His_6 tag. Similarly, Pfp23 expressed from the recombinant pET24a vector harbored a C-terminal His_6 tag. The molecular weights of the recombinant proteins are approximately 114 kDa (PfHsp90 from pGK), 88 kDa (PfHsp90 from pET24a) and 36 kDa (Pfp23). *M* protein marker (Biorad)

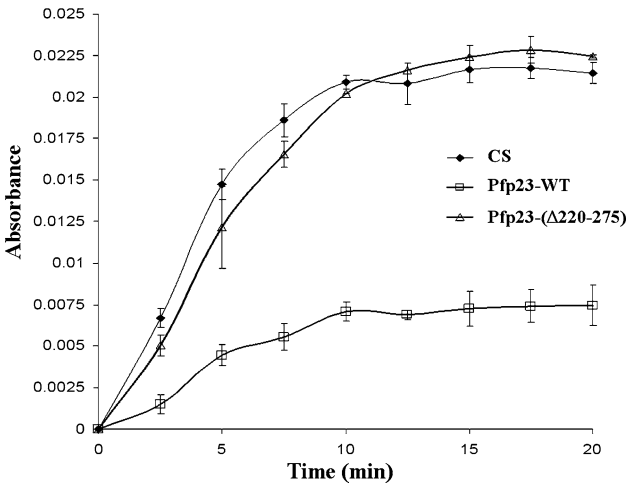

Fig. 3 The Pfp23 recombinant protein exhibited intrinsic chaperone activity. The intrinsic chaperone activity of Pfp23 was determined by citrate synthase aggregation assay. Spontaneous aggregation of citrate synthase was monitored continuously over 20 min at 45°C by the increase in absorbance. Addition of Pfp23-WT suppressed the aggregation of citrate synthase. The anti-aggregation activity was abolished when Pfp23-WT was substituted with Pfp23-(Δ220–275), which has a truncated C-terminal tail. Results shown are representative of three independent experiments. *CS* citrate synthase

experiments performed without citrate synthase (data not shown).

Pfp23 interaction with PfHsp90 is dependent on $MgCl_2$ and ATP

The role of Pfp23 as a co-chaperone of PfHsp90 in *P. falciparum* has remained debatable as previous attempts to identify their interaction via co-immunoprecipitation techniques were unsuccessful [20, 23]. As such, their interaction was probed in this study using recombinant proteins and GST pull-down assays. Purified Pfp23 recombinant protein was added to PfHsp90 immobilized on glutathione Sepharose in a 5:1 ratio and incubated either in the presence or absence of 5 mM $MgCl_2$, 5 mM ADP or 5 mM ATP. Under the different conditions tested, the binding of Pfp23 to PfHsp90 was only observed in the presence of $MgCl_2$ and ATP (Fig. 4). Recombinant GST-His_6 fusion protein without PfHsp90 was used as a control to ensure that the recombinant tags do not contribute to the interaction observed. Pfp23 binding was absent in the control experiments performed under similar conditions (data not shown).

Sequential N-terminal truncation of Pfp23 affects interaction with PfHsp90

As Pfp23 shares low amino acid sequence identities with the human p23 and yeast SbaI, its interaction with PfHsp90 may potentially differ from that of their homologues.

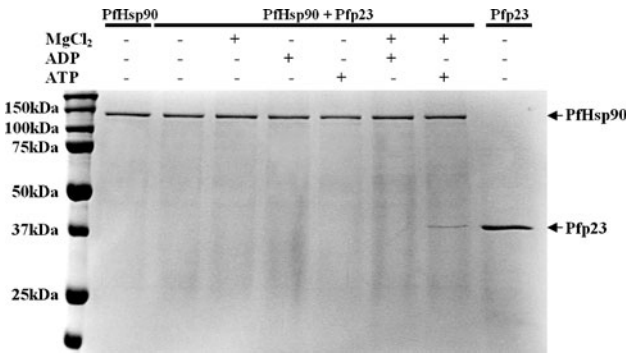

Fig. 4 SDS–PAGE analyses of Pfp23 interaction with PfHsp90. The interaction between Pfp23 and PfHsp90 was tested in the presence or absence of 5 mM $MgCl_2$, 5 mM ADP and 5 mM ATP as indicated. Pfp23 was bound to PfHsp90 only in the presence of $MgCl_2$ and ATP. Results shown are representative of three independent experiments

Hence, the interaction between Pfp23 and PfHsp90 was further analyzed. Pfp23 was sequentially truncated by 25 amino acid residues from the N-terminal end to identify regions that are essential for binding PfHsp90 (Fig. 5a).

A total of five Pfp23-truncated mutants (T1 to T5) were constructed, cloned, expressed and purified under similar conditions to those described for Pfp23-WT. All the truncated mutants were solubly expressed in *E. coli* BL21 (DE3) (data not shown). Pfp23-truncated mutants T1, T2 and T3 retained their binding to PfHsp90, whereas further truncations in T4 and T5 abolished interaction with PfHsp90 (Fig. 5b). The results indicated that certain amino acid residues located between the 75th to 100th positions in

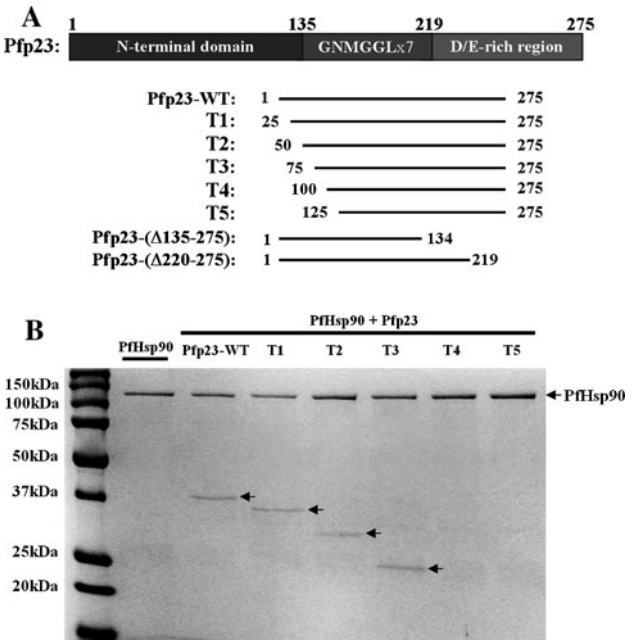

Fig. 5 Interaction between Pfp23-WT and truncated mutants with PfHsp90. **a** Schematic representation of the Pfp23-truncated mutants constructed in this study. A 25-amino acid serial truncation was performed from the N-terminal end of Pfp23 (T1 to T5) to identify the region that is essential for binding PfHsp90. Pfp23-(Δ220–275) was constructed to investigate the intrinsic chaperone activity of Pfp23. On the other hand, Pfp23-(Δ135–275) was generated to determine whether the **GNMGGL**x7 sequence repeats are essential for PfHsp90 interaction. **b** Purified Pfp23-WT and the respective truncated mutants were incubated with PfHsp90 in the presence of 5 mM $MgCl_2$ and 5 mM ATP. Truncated mutants T4 and T5 were unable to bind PfHsp90, as shown in the SDS-PAGE analysis. Results presented are representative of three independent experiments

Pfp23 are crucial for binding PfHsp90. To identify these amino acid residues, the corresponding region in yeast SbaI was determined by primary sequence alignment and examined for the presence of Hsp82-interacting residues. Inspection of the region using the yeast Hsp82-SbaI crystal structure complex revealed an interface in SbaI (K113, Y114, P115, Y116, I117 and K118) that interacts with the closed 'lid' segment of Hsp82 (Fig. 6a) [14]. This interface corresponded to the amino acid residues K91, K92, H93, W94, V95 and K96 in Pfp23 and was observed to be in proximity to PfHsp90 in the modeled complex (Fig. 6b). Hence, these residues may potentially be essential for PfHsp90 interaction, such that when deleted (in Pfp23-truncated mutants T4), Pfp23's competency in binding PfHsp90 is undermined.

Residues K91, H93, W94 and K96 in Pfp23 are essential for PfHsp90 interaction

To investigate the importance of the amino acid residues K91, K92, H93, W94, V95 and K96 in Pfp23 for PfHsp90 interaction, single mutants were generated. Firstly, Pfp23

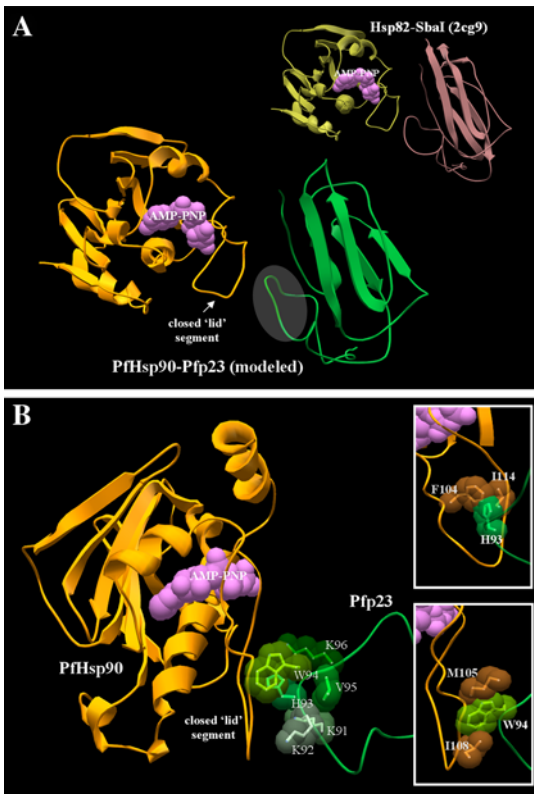

Fig. 6 Analyses of the modeled structures of Pfp23 and PfHsp90. **a** PfHsp90 (*orange*) and Pfp23 (*green*) were modeled against the yeast Hsp82 (*gold*)-SbaI (*brown*) crystal structure complex (PDB accession number: 2cg9). The modeled Pfp23 shares similar structural features with SbaI and possesses an extended loop (*highlighted in grey*) that is projected towards the closed 'lid' segment of PfHsp90. Only the N-terminal domains of PfHsp90 and Hsp82 were shown in the graphical presentation. **b** Amino acid residues K91, K92, H93, W94, V95 and K96, located at the extended loop of Pfp23, were observed to be in proximity to the closed 'lid' segment of PfHsp90. Pfp23 residue H93 was observed to be in close contact with the hydrophobic residues F104 and I114 in PfHsp90 (*top right*). Similarly, the residue W94 in Pfp23 is directed towards residues I108 and M105 in PfHsp90 (*bottom right*). *AMP-PNP* a non-hydrolysable analogue of ATP

residues K91, K92 and K96 were each mutated to an alanine to examine the importance of the lysine side chains for binding PfHsp90. As for residues H93 and W94, mutation to a polar serine residue was proposed to disrupt any hydrophobic interaction formed between Pfp23 and PfHsp90. Similarly, the hydrophobic amino acid residue, V95, was mutated to a polar asparagine residue as a previous study showed that substitution at the corresponding position in SbaI (I117N) abolished interaction with Hsp82 [35]. All the Pfp23 mutant proteins were solubly expressed, purified and tested for PfHsp90 interaction under similar binding conditions to those described for Pfp23-WT. Pfp23 mutants K91A, H93S, W94S and K96A displayed significant reduction in their binding to PfHsp90, retaining less than 25% of Pfp23-WT's binding (Fig. 7). However, Pfp23 mutants K92A and V95 N retained more than 85% of

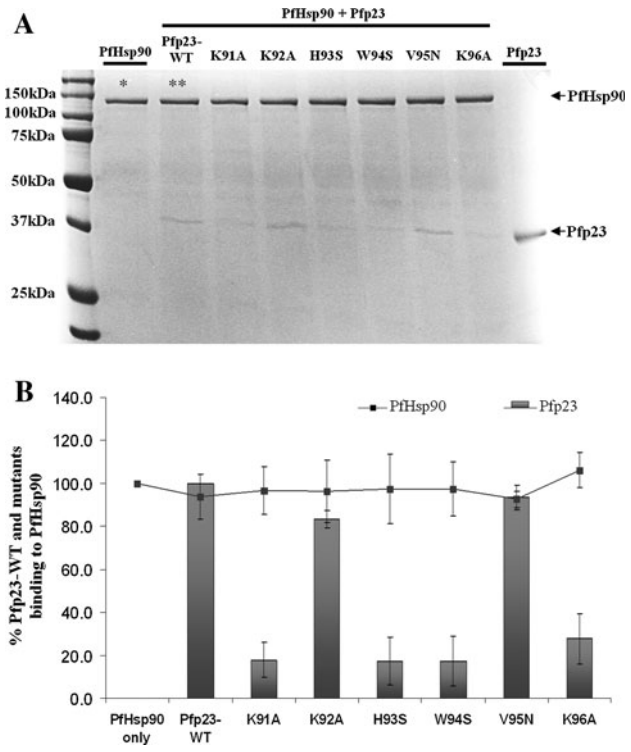

Fig. 7 Effects of Pfp23 mutations on PfHsp90 interaction. **a** Pfp23-WT and mutants were incubated with PfHsp90 in the presence of 5 mM $MgCl_2$ and 5 mM ATP. The complexes formed were analyzed by SDS-PAGE. **b** The binding of Pfp23 mutants to PfHsp90 was analyzed by densitometry. The percentage of PfHsp90 bound was calculated with respect to the PfHsp90 control (*single asterisk*), whereas Pfp23 mutants binding to PfHsp90 was determined with respect to Pfp23-WT (*double asterisk*). Pfp23 single mutants K91A, H93S, W94S and K96A displayed drastically reduced binding to PfHsp90, whereas single mutants K92A and V95 N retained binding comparable to Pfp23-WT to PfHsp90. Results shown are representative of three independent experiments

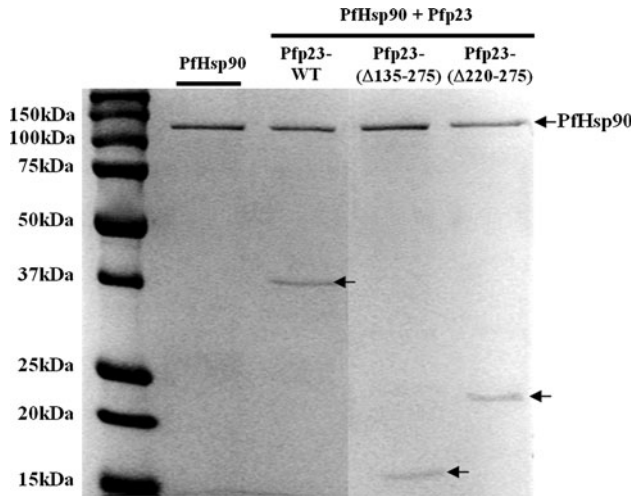

Fig. 8 SDS-PAGE analyses of Pfp23-(Δ220–275) and Pfp23-(Δ135–275) interactions with PfHsp90. To investigate the importance of the **GNMGGL**x7 sequence repeats in Pfp23 for PfHsp90 interaction, Pfp23-(Δ135–275) was constructed. Using Pfp23-(Δ220–275) mutant, the C-terminal tail of Pfp23 was shown to be dispensable for PfHsp90 interaction. Further deletion of the **GNMGGL**x7 sequence repeats in Pfp23-(Δ135-275) did not affect its binding to PfHsp90, indicating that the sequence repeats are not essential for PfHsp90 interaction

Pfp23-WT's binding to PfHsp90. Recombinant GST-His_6 fusion protein without PfHsp90 was used as a control, and no interaction with Pfp23 mutants was observed when tested under similar conditions (data not shown).

The GNMGGLx7 sequence repeats in Pfp23 are not essential for PfHsp90 interaction

The protein sequence of Pfp23 contains **GNMGGL**x7 sequence repeats, which have yet to be functionally characterized. To determine if the sequence repeats are important for PfHsp90 interaction, two additional truncated mutants, Pfp23-(Δ220–275) and Pfp23-(Δ135–275), were constructed. The Pfp23-(Δ220–275) mutant contained the **GNMGGL**x7 sequence repeats but lacked the C-terminal tail, whereas Pfp23-(Δ135–275) had both the sequence repeats and the C-terminal tail deleted (Fig. 8). SDS-PAGE analysis showed that Pfp23-(Δ220–275) retained interaction with PfHsp90. Similarly, further removal of the **GNMGGL**x7 sequence repeats in Pfp23-(Δ135–275) did not affect the mutant's competency in binding PfHsp90. Hence, the results indicated that the **GNMGGL**x7 sequence repeats are not essential for PfHsp90 interaction.

Pfp23 inhibits the ATPase activity of PfHsp90

The binding and hydrolysis of ATP are known to induce conformational changes in Hsp90, essential for the chaperoning of its client proteins [3–5]. To determine whether the binding of Pfp23 affects the hydrolysis of ATP in PfHsp90, and henceforth, the regulation of its chaperone function, ATPase assay was carried out.

Using a sensitive enzyme-coupled assay, PfHsp90 was shown to have an ATPase activity of 0.504 ± 0.044 $\mu mol_{ATP}/min/\mu mol_{PfHsp90}$ at 37°C. The activity measured was sensitive to inhibition by radicicol, a specific Hsp90 ATPase inhibitor, and thus ensured that the activity detected was not attributed to other co-purified ATPase contaminants. The activity observed is approximately ten times more than the human Hsp90 ATPase activity (0.034–0.089 $\mu mol_{ATP}/min/\mu mol_{Hsp90}$) [10, 36], but comparable to that reported for yeast Hsp82 (0.40–1.05 $\mu mol_{ATP}/min/\mu mol_{Hsp82}$) [5, 6, 37, 38].

The effect of Pfp23 interaction on PfHsp90 ATPase activity was subsequently investigated by the addition of Pfp23 in increasing concentrations to the assay reactions (Fig. 9a). Introduction of an equimolar concentration of Pfp23 had a negligible effect on the ATPase activity of

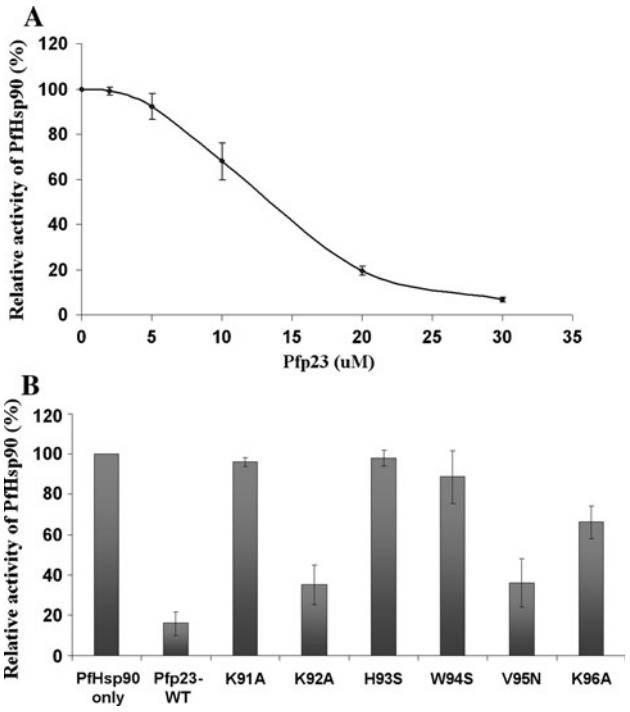

Fig. 9 Effects of Pfp23-WT and mutants on PfHsp90 ATPase activity. **a** Pfp23 was added in increasing concentrations to the ATPase assay reactions containing a fixed amount of PfHsp90 (2 μM). The ATPase activity of PfHsp90 was inhibited by Pfp23 in a concentration-dependent manner. At ten-fold molar excess, Pfp23 inhibited the ATPase activity of PfHsp90 to approximately 20% of its original activity. **b** Pfp23 single mutants were tested for their ability to inhibit the ATPase activity of PfHsp90. Pfp23 single mutants known to have reduced binding to PfHsp90 (K91A, H93S, W94S and K96A) displayed a negligible inhibitory effect on its ATPase activity. However, Pfp23 single mutants (K92A and V95 N) that exhibited comparable binding to PfHsp90 as Pfp23-WT inhibited PfHsp90 ATPase activity to approximately 35% of its original activity. Pfp23-WT and mutants were added in ten-fold molar excess to PfHsp90 in all the assay reactions. Results shown are representative of two independent experiments

PfHsp90. However, when added in ten-fold molar excess, Pfp23 reduced the ATPase activity of PfHsp90 to approximately 20% of its original activity.

To further verify whether the ATPase inhibitory effect was due to the interaction of Pfp23 with PfHsp90, Pfp23 single mutants with differential PfHsp90 binding levels were used. As expected, Pfp23 single mutants (K91A, H93S, W94S and K96A), which displayed drastically reduced PfHsp90 binding, had little inhibitory effect on the ATPase activity of PfHsp90 (Fig. 9b). PfHsp90 retained approximately 90% of its ATPase activity in the presence of Pfp23 single mutants K91A, H93S and W94S, respectively. Nevertheless, Pfp23 single mutant K96A was able to suppress the ATPase activity of PfHsp90 to approximately 65% of its original activity. On the contrary, Pfp23 single mutants (K92A and V95 N) that have a binding capacity to PfHsp90 similar to Pfp23-WT significantly inhibited the ATPase activity of PfHsp90 to approximately 35% of its original activity. The results clearly demonstrated the inhibitory effect that Pfp23 binding has on the ATPase activity of PfHsp90, and this may have significance in the regulation of PfHsp90 chaperone function in vivo.

Discussion

Treatment of malaria is increasingly challenging with the emergence of new strains that are resistant to conventional anti-malaria drugs such as chloroquine and pyrimethamine-sulfadoxine [39, 40]. Coupled with the lack of efficacious vaccines, there is a need to search for new anti-malarials and drug targets. Recent studies have recognized the potential of targeting PfHsp90 for malaria therapy as its inhibition effectively arrests the intra-erythrocytic development of *P. falciparum* [23, 41, 42]. Nevertheless, there are some considerations on the use of Hsp90-specific inhibitors as anti-malarials. How selective inhibition of PfHsp90 can be achieved in humans remains a concern since the Hsp90s in the two organisms are fairly conserved, with 63% amino acid identity [43]. Furthermore, the paucity of knowledge on the Hsp90 chaperone system in *P. falciparum* hinders the understanding of its function in the malaria parasite. Hence, identifying factors that regulate the function of PfHsp90 and its affinity for the inhibitors is imperative to facilitate the assessment of its relevance as a drug target.

One of the factors that influences Hsp90 activity and sensitivity to the inhibitors is the binding of co-chaperones [44–48]. However, thus far, PfHsp90 has only been demonstrated to form complexes with *P. falciparum* Hsp70, PP5, PKBP35 and α-tubulin [23, 49, 50]. It is surprising that homologues of co-chaperones (e.g., p23, Hop), which are known to be essential in regulating the chaperone function of Hsp90 in human and yeast [2], were not detected in those PfHsp90 complexes isolated. Inevitably, this has raised doubts about the functional conservation of these putative co-chaperones in *P. falciparum*. As the interaction between p23 and Hsp90 is essential for client protein activation, and recently has been shown to influence the sensitivity of yeast to Hsp90-specfic inhibitors [44], the interaction status of their homologues in *P. falciparum* may have implications on the modus operandi of PfHsp90 and its affinity for the drugs. Hence, in this study, the putative co-chaperone Pfp23 was investigated for its functionality and interaction with PfHsp90.

The purified Pfp23 recombinant protein was shown to possess intrinsic chaperone activity by virtue of its ability to suppress the aggregation of citrate synthase at 45°C. However, removal of the C-terminal tail of Pfp23 resulted in the loss of this anti-aggregation activity and attests to the need to have a full-fledged Pfp23 protein to elicit the

chaperone activity. Pfp23 was subsequently probed for its interaction with PfHsp90 to assess its potential role as a co-chaperone in *P. falciparum*. Using GST pull-down assays, Pfp23 was observed to bind PfHsp90 in the presence of $MgCl_2$ and ATP. The conditions conducive for binding were similar to that of the human and yeast homologues [7, 8], where the Mg^{2+} ion is essential as a cofactor for ATP binding in Hsp90 [51]. Truncation experiments indicated that the unique **GNMGGL**x7 sequence repeats in Pfp23 are dispensable for PfHsp90 interaction. Instead, when coupled to computational analyses, the results from the truncation study suggested that the 91st to 96th amino acid residues in Pfp23 may form an essential interface for binding PfHsp90. This interface (consisting of amino acid residues K91, K92, H93, W94, V95 and K96) is located at an extended loop of Pfp23 and was observed to be in proximity to the closed 'lid' segment of PfHsp90 (Fig. 6a). Site-directed mutagenesis experiments affirmed the importance of residues K91, H93, W94 and K96 for PfHsp90 interaction. Their respective single amino acid substitutions, K91A, H93S, W94S and K96A, resulted in drastically reduced binding to PfHsp90. However, Pfp23 mutants with K92A or V95 N substitution displayed PfHsp90 binding levels comparable to Pfp23-WT. The effects of these mutations were analyzed by computationally modeled structures to further comprehend the molecular forces that are involved in their interaction.

With the aid of modeled tertiary structures, the side chains of K91, H93, W94 and K96 in Pfp23 were observed to be projected towards the closed 'lid' segment of PfHsp90 (Fig. 6b). Under the in vitro binding conditions used (at pH 7.5), the side chain of the histidine residue (H93) would be uncharged, suggesting that the main force of interaction is hydrophobic in nature. This was supported by the observation that its side chain is located close to the hydrophobic residues F104 and I114, of PfHsp90 (Fig. 6b). Similar hydrophobic interaction would be expected of Pfp23 residue W94, which was visualized to be in proximity to I108 and the aliphatic side chain of M105 in PfHsp90. Conceivably, substitutions of H93 and W94 to a polar serine residue, respectively, would disrupt the hydrophobic interaction and result in the diminished binding to PfHsp90, as observed in this study.

Apart from that, *in silico* analyses of the Pfp23 residues, K91 and K96, did not reveal any electrostatic interaction between the charged group in the lysine side chains with PfHsp90 residues. This is consistent with the observation that the interaction between Pfp23 and PfHsp90 was stable at high salt concentrations of up to 300 mM KCl (data not shown), suggesting that electrostatic force plays a minor role in their interaction. Instead, results from the mutation study suggest that the long aliphatic side chains of the lysine residues may be important in contributing to the hydrophobic interaction between the Pfp23 residues, H93 and W94, with PfHsp90. Substitution to an alanine with a much shorter side chain would greatly diminish the hydrophobicity and proximity required for binding PfHsp90, and this may account for the lack of PfHsp90 interaction as observed.

On the contrary, the Pfp23 mutant K92A retained a similar PfHsp90 binding capacity to that of the Pfp23-WT. This implies that the residue K92 is not essential for PfHsp90 interaction. Likewise, replacing V95 with an asparagine resulted in a mutant that retained comparable binding to PfHsp90 as the Pfp23-WT. This result is surprising as the corresponding mutation in yeast SbaI (I117N) had rendered it defective in binding Hsp82 [35]. However, using the modeled structures, the side chain of V95 was observed to be projected away from PfHsp90. In view of this, Pfp23 mutant V95N's ability to retain its binding to PfHsp90 is likely to be attributed to the lack of interaction between the amino acid residue V95 and PfHsp90.

The results of the truncation study have also highlighted that a large segment of Pfp23's N-terminal domain (1st to 74th amino acid residues) can be deleted without affecting its interaction with PfHsp90. This segment corresponds to the region in SbaI (1st to 93rd amino acid residues) where three other Hsp82-interacting interfaces (amino acid residues 13–16, 31–37, 85–91) were found [14]. It is difficult to postulate whether the deletion of these interfaces in SbaI (similar to that of Pfp23-truncated mutants T4) would affect Hsp82 interaction, as only a few amino acid residues located at these interfaces were conserved at the corresponding positions in Pfp23.

Probing the significance of Pfp23 binding on PfHsp90 function, results from the ATPase assays revealed that Pfp23 is capable of suppressing the hydrolysis of ATP by PfHsp90. The ATPase inhibitory effect of Pfp23 is positively correlated with its binding capacity to PfHsp90. Pfp23 single mutants (K91A, H93S, W94S and K96A) that were defective in binding PfHsp90 were unable to inhibit PfHsp90 ATPase activity as effectively as Pfp23-WT. The observation is consistent with that reported for the human and yeast homologues [10, 11, 52], and it was suggested that the binding of p23 traps Hsp90 in the ATP-bound state and prolongs its interaction with the client proteins. Nevertheless, it is noteworthy that the ATPase inhibitory effect was only observed when Pfp23 was added in large molar excess. While this may indicate limitations of the in vitro environment for stable binding of Pfp23 to PfHsp90, it may also highlight the transient interaction between the two proteins. Coupled with semi-quantitative mass spectrometry data that suggested that Pfp23 is present at a low physiological concentration in *P. falciparum* [53, 54], these observations could explain why

previous studies were unsuccessful in identifying the PfHsp90-Pfp23 complex through co-immunoprecipitation techniques [20, 23].

With the recognition of PfHsp90 as a potential drug target against malaria, there is increasing interest in the development of assays for testing Hsp90-specific inhibitors as anti-malarials. In a recent study, PfHsp90 was shown to be capable of supporting the survival of a yeast strain with both Hsp90 genes (*Hsc82* and *Hsp82*) deleted, which is otherwise lethal [42]. This has led to the proposal of exploiting the viable yeast strain complemented with PfHsp90 to screen for inhibitors that are selective against the parasite Hsp90. While this method offers the advantage of an in vivo setting for drug screening, the slower growth profile reported may reflect non-optimal heterologous interaction between PfHsp90 and the yeast co-chaperones and client proteins. Since the binding of co-chaperones has a major influence on the affinity of Hsp90 for the inhibitors [44–48], it alerts us to the need to assess the sensitivity of PfHsp90 to the inhibitors in the presence of native *P. falciparum* co-chaperones, using a priori an in vitro assay system. Providing an experimentally endorsed PfHsp90 chaperone complex is important to set the stage correctly for testing inhibitors against this protein consortium in *P. falciparum*. To this end, it is thus essential to determine and validate experimentally the interaction between PfHsp90 and its putative co-chaperones, particularly that of p23 with its underscored regulatory function and influence on Hsp90's affinity for the inhibitors [44, 55].

Taken together, this study demonstrated that the *P. falciparum* genome encodes a functional p23 homologue and has provided valuable evidence concerning its in vitro interaction with PfHsp90. The inhibitory effect that Pfp23 exerts on the ATPase activity of PfHsp90 further highlighted its potential regulatory role on the chaperone function of PfHsp90 in vivo. In addition, truncation experiments and site-directed mutagenesis demonstrated significant roles played by certain amino acid residues in Pfp23, i.e., K91, H93, W94 and K96 in PfHsp90 interaction. Analyses of the modeled structures further revealed these residues to be strategically located at an extended loop of Pfp23 that presumably interacts with the closed 'lid' segment of PfHsp90 via hydrophobic forces. As the interaction between Pfp23 and PfHsp90 was not observed in previous studies [20, 23], this may underline the technical difficulties in using co-immunoprecipitation to identify PfHsp90 complexes. In view of this, a similar GST pull-down approach would be useful and may be adopted to investigate the interactions between PfHsp90 and other putative co-chaperones (e.g., Hop), which were also not detected in previous studies.

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