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Molecular analysis of *Plasmodium falciparum* co-chaperone Aha1 supports its interaction with and regulation of Hsp90 in the malaria parasite

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ABSTRACT

The recent recognition of *Plasmodium falciparum* Hsp90 (PfHsp90) as a promising anti-malaria drug target has sparked interest in identifying factors that regulate its function and drug-interaction. Co-chaperones are well-known regulators of Hsp90's chaperone function, and certain members have been implicated in conferring protection against lethal cellular effects of Hsp90-specific inhibitors. In this context, studies on PfHsp90's co-chaperones are imperative to gain insight into the regulation of the chaperone in the malaria parasite. In this study, a putative co-chaperone *P. falciparum* Aha1 (PfAha1) was identified and investigated for its interaction and regulation of PfHsp90. A previous genome-wide yeast two-hybrid study failed to identify PfAha1's association with PfHsp90, which prompted us to use a directed assay to investigate their interaction. PfAha1 was shown to interact with PfHsp90 via the *in vivo* split-ubiquitin assay and the association was confirmed *in vitro* by GST pull-down experiments. The GST pull-down assay further revealed PfAha1's interaction with PfHsp90 to be dependent on $MgCl_2$ and ATP, and was competed by co-chaperone Pfp23 that binds PfHsp90 under the same condition. In addition, the PfHsp90–PfAha1 complex was found to be sensitive to disruption by high salt, indicating a polar interaction between them. Using bio-computational modelling coupled with site-directed mutagenesis, the polar residue N108 in PfAha1 was found to be strategically located and essential for PfHsp90 interaction. The functional significance of PfAha1's interaction was clearly that of exerting a stimulatory effect on the ATPase activity of PfHsp90, likely to be essential for promoting the activation of PfHsp90's client proteins.

1. Introduction

Protein folding and activation mediated by the molecular chaperone Hsp90 (Heat shock protein 90) is a complex ATP-driven process that is regulated by a cohort of accessory proteins termed co-chaperones (reviewed in Pearl and Prodromou, 2006; Neckers et al., 2009; Taipale et al., 2010). A subset of these co-chaperones (e.g. Hop, p23, and Aha1) coordinates the progression of Hsp90's chaperone cycle by modulating the ATPase activity of Hsp90 that is tightly coupled to its conformational states (Wandinger et al., 2008; Hessling et al., 2009; Li et al., 2011). The co-chaperone Aha1 (Activator of Hsp90 ATPase) is known to associate with Hsp90 and activate its ATPase activity to promote the activation of Hsp90's client protein.

Aha1 was initially discovered as a homologue of *Saccharomyces cerevisiae* Hch1, a suppressor of growth defect observed in temperature sensitive yeast strain carrying mutant Hsp82 (Hsp90 in yeast) with E381K substitution (Nathan et al., 1999; Panaretou et al., 2002). Gene deletion studies in yeast indicated that both Aha1 and Hch1 are non-essential for viability, although temperature sensitive phenotypes were observed in the single and double deletion-strains constructed i.e. Δ*AHA1*, Δ*HCH1* and Δ*AHA1*–Δ*HCH1* (Panaretou et al., 2002; Lotz et al., 2003). Nevertheless, the deletion of Aha1 significantly impaired the activation of glucocorticoid receptor and v-Src kinase in yeast, suggesting that the presence of the co-chaperone is essential for functional maturation of these Hsp82 client proteins (Lotz et al., 2003; Meyer et al., 2003). Similar observation has been made in an siRNA study conducted on mammalian cell culture where downregulation of Aha1 led to drastically reduced level of activated glucocorticoid receptors (Harst et al., 2005). Together with the observed role in activating Hsp90's ATPase activity, Aha1 has been proposed as a positive regulator of Hsp90's chaperone function, whose association with Hsp90 is important in driving certain client proteins towards their activated states.

Abbreviations: 5-FOA, 5-fluoroorotic acid; ADP, adenosine diphosphate; Aha1, Activator of Hsp90 ATPase 1; ATP, adenosine triphosphate; C_{ub}, C-terminal half of ubiquitin; DNA, deoxyribonucleic acid; GST, glutathione *S*-transferase; His_6, hexa-histidine; Hsp90, Heat shock protein 90; Ni-NTA, nickel-nitrilotriacetic acid; NMR, nuclear magnetic resonance; N_{ub}, N-terminal half of ubiquitin; PCR, polymerase chain reaction; PDB, Protein Data Bank; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WT, wild-type.

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In *Plasmodium falciparum*, a putative Aha1 (PfAha1; PlasmoDB accession number: PFC0270w) that shares approximately 20% amino acid sequence identity with that of the yeast homologue has been described in a computational study (Kumar et al., 2007). As the putative PfAha1 has not been characterized prior to this study, its role as a co-chaperone of *P. falciparum* Hsp90 (PfHsp90) remains elusive. PfHsp90 has recently emerged as a promising drug target for malaria inhibition (Pesce et al., 2010; Shonhai, 2010) as its specific inhibitors have been shown to cause growth arrests in *P. falciparum* intra-erythrocytic stage cultures (Banumathy et al., 2003; Kumar et al., 2003). This indicates PfHsp90's involvement in crucial biological pathways in *P. falciparum* and thereafter its inhibition, an attractive avenue to abrogate the cellular activities of the parasite. Despite recognizing the importance of PfHsp90 for the survival of the parasite, knowledge on the chaperone function and regulation of PfHsp90 remains limited, partly due to difficulties in genetic manipulation of the Plasmodium parasite. So far, co-immunoprecipitation and yeast two-hybrid experiments were unable to provide evidence on the association between PfHsp90 and many putative co-chaperones (e.g. Aha1, Hop, and p23) that are known to play essential roles in the yeast and human Hsp90 chaperone cycle (Banumathy et al., 2003; LaCount et al., 2005). The interaction between *P. falciparum* p23 (Pfp23) and PfHsp90 was only recently elucidated using recombinant proteins and GST pull-down approach, whereby Pfp23 reportedly exerts an inhibitory effect on the ATPase activity of PfHsp90 (Chua et al., 2010). In view of the importance of Aha1 in the yeast and human Hsp90 chaperone system for client protein activation, the putative PfAha1 is investigated in this study with the intention of characterizing its interaction and regulation of PfHsp90.

2. Materials and methods

2.1. Split-ubiquitin assay

The use of the split-ubiquitin system for investigating protein interaction in yeast has been described previously (Lehming, 2002; Laser et al., 2000). PfHsp90 (PF07_0029) and PfAha1 (PFC0270w) were reverse transcribed and amplified from the mRNA extracted from *P. falciparum* 3D7 (MRA-102, MR4, ATCC Manassas, VA) and subsequently used as a template to clone the respective PfHsp90 (PfHsp90N, PfHsp90M and PfHsp90C) and PfAha1 (PfAha1N and PfAha1C) domains using appropriate forward and reverse primers (Table 1). PfHsp90N, PfHsp90M and PfHsp90C DNA products were digested with *EcoR*I and *Sal*I and ligated into the C_{ub}-RUra3p vector (Wittke et al., 1999), whereas PfAha1, PfAha1N and PfAha1C were digested with *Bgl*II and *Sal*I and subsequently cloned into the N_{ub} vector (Laser et al., 2000). The recombinant plasmids were transformed into *Escherichia coli* DH5α cells and their authenticity were verified by DNA sequencing.

To investigate the interaction between PfHsp90 and PfAha1, the appropriate PfHsp90-C_{ub}-RUra3p and N_{ub}-PfAha1 fusion constructs were co-transformed into the *S. cerevisiae* strain *JD52* and plated onto media lacking leucine and tryptophan to select for transformants that carried both constructs. Subsequently, the yeast cells that contained both the recombinant PfHsp90-C_{ub}-RUra3p and N_{ub}-PfAha1 fusion constructs were tenfold serial-diluted and dropped onto plates lacking leucine and tryptophan, onto plates additionally lacking uracil and onto plates additionally containing 5-fluoroorotic acid (5-FOA).

2.2. Construction of PfHsp90 and PfAha1 plasmids for recombinant protein expression

The molecular cloning of PfHsp90 into expression vectors pGK and pET24a (Novagen) has been reported (Chua et al., 2010). For cloning of PfAha1 into bacterial expression vector, PfAha1 was re-amplified by PCR from the N_{ub}-PfAha1 recombinant plasmid using appropriate forward and reverse primers (Table 1), digested with *Bam*HI and *Xho*I, and ligated into the expression vector pET24a. The ligated recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells that contained a RIG plasmid (Baca and Hol, 2000) for recombinant protein expression. DNA sequencing was performed to authenticate the recombinant clone that carries *PfAha1*.

Table 1
Primers designed for gene cloning and mutations.

Gene constructs	Primer sequence[a] (5′–3′)
PfAha1 (PFC0270w)	F: ACGggatccATGTCAGGATCAGTATGG R: ACGctcgagTTTTTCTCTCATTTTTAAAATAAAATCATC
Split-ubiquitin assay constructs	
PfHsp90	F: gaattcAAAATGTCAACGGAAACATTCGC R: gtcgacCCGTCAACTTCTTCCATTTTAGAATCGG
PfHsp90N (1–220)	F: gaattcAAAATGTCAACGGAAACATTCGC R: gtcgacCCAGATGCGGTGATTTC
PfHsp90M (295–580)	F: gaattcAAAATGAGAAAAAAAAAAAATACACACAG R: gtcgacCCGAAATCTTTTTTGGCTTCTTCTG
PfHsp90C (581–745)	F: gaattcAAAATGGAAACCTTGAAAGCTGAATATGAAGG R: gtcgacCCGTCAACTTCTTCCATTTTAGAATCGG
Aha1	F: AGTAGTagatctTGATGTCAGGATCAGTATGG R: CATCATgtcgacTTATTTTTCTCTCATTTTTAAAATAAAATC
Aha1N (1–155)	F: AGTAGTagatctTGATGTCAGGATCAGTATGG R: CATCATgtcgacTTACTTATTAGATTCATTTTTATCG
Aha1C (156–349)	F: AGTAGTagatctTGGAGTTAAAAATTAAAGAGG R: CATCATgtcgacTTATTTTTCTCTCATTTTTAAAATAAAATC
PfAha1 mutations[b]	
E15A	F: CACTGGGAA**GCA**AGAAATTACAATAAATGG R: CATTTATTGTAATTTCT**TGC**TTCCCAGTGC
R58A	F: GCTTGTGTTTCCATA**GCA**AAGGGGAAAC R: GTTTCCCCTT**TGC**TATGGAAACACAAGC
E67A	F: TT**GCA**TATATTATAAAATTTGAATGG R: ATAATATA**TGC**AAAAGAATTTATTTGTTTC
D94A	F: GGTTGAAATTCCG**GCT**TTTTCTACATTTTC R: GAAAATGTAGAAAA**AGC**CGGAATTTCAACC
K127A	F: ATGATAGCATATTA**GCA**AAGGAAGGCAAGG R: CCTTGCCTTCCTT**TGC**TAATATGCTATCAT
E91A	F: GGTTCGGTT**GCA**ATTCCGGATT R: GGAAT**TGC**AACCGAACCACC
F98A	F: GATTTTTCTACA**GCT**TCTTTAGAGG R: CTAAAGA**AGC**TGTAGAAAAATCCG
F98A/D94A	F: **GCT**TTTTCTACA**GCT**TCTTTAGAGG R: CTAAAGA**AGC**TGTAGAAAA**AGC**CG
N108A	F: TTATGCTATA**GCT**ATTGAGCGAAC R: GTTCGCTCAAT**AGC**TATAGCATAATC
E110A/R111A	F: TATAAATATT**GCGGCA**ACAGATGAATCG R: ATTCATCTGT**TGCCGC**AATATTTATAGC
R119A	F: GAAAACTTA**GCA**TTTATATATGATAG R: CATATATAAA**TGC**TAAGTTTTCCGAT

[a] Restriction enzyme sites are in lowercase; F represents forward primer; R represents reverse primer.
[b] Codons mutated in PfAha1 are in bold and underlined.

2.3. Expression and purification of PfHsp90, Pfp23 and PfAha1 recombinant proteins

The expression and purification of PfHsp90 and Pfp23 recombinant proteins have been described (Chua et al., 2010). An identical protocol used for the expression and purification of Pfp23 was adopted for PfAha1 since they were cloned into the same