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15-Deoxyspergualin modulates *Plasmodium falciparum* heat shock protein function ☆

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Abstract

Heat shock proteins are essential for the survival of all cells. The C-terminal EEVD motif of Hsp70 has previously been implicated in binding 15-deoxyspergualin (DSG), an immunosuppressant with antimalarial activity whose mechanism of action is uncertain. We report the cloning, overexpression, and characterization of three members of the heat shock family, PfHsp70-1 (an Hsp70 protein with a C-terminal EEVD motif), PfHsp70-2 (an Hsp70 protein without the EEVD motif), and PfHsp70 interacting protein. The chaperone activity of PfHsp70-1, and PfHsp70-2 was enhanced by ATP and by PfHip. Interestingly, while binding of protein substrates to PfHsp70-1, PfHsp70-2 and PfHip was unaffected in the presence of DSG, the ATP enhanced chaperone activity of PfHsp70-1 but not PfHsp70-2 was stimulated further by DSG. Our finding suggests that the binding partner of DSG in the parasite cellular milieu is PfHsp70-1 and paves the way for the elucidation of the mechanism of antimalarial action of DSG.

Keywords: Hsp70; Hsp70 interacting protein; *Plasmodium falciparum*; Chaperone activity; 15-Deoxyspergualin

Heat shock proteins, often termed the molecular chaperones [1], are evolutionarily highly conserved and ubiquitous proteins essential to the cellular homeostasis of all prokaryotes and eukaryotes. They ensure the stability of other cellular proteins in normal conditions and protect them from various physiological and environmental insults. Heat shock proteins also fulfill a variety of functions such as intracellular assembly, folding and translocation of oligomeric proteins, import of proteins into cellular compartments, folding of proteins in the cytosol, endoplasmic reticulum, and mitochondria, degradation of unstable proteins, dissolution of protein complexes, prevention of protein aggregation, refolding of misfolded proteins, control of regulatory proteins such as transcription factors, replication proteins, and kinases, protein signaling including steroid hormone activation and tumor immunogenicity, and antigen presentation [2,3]. Heat shock proteins are localized in different cellular compartments and have been classified based on the approximate molecular mass [4].

The 70-kDa heat shock protein family (Hsp70) is a well-characterized family of heat shock proteins belonging to various subcellular compartments and combating diverse forms of cellular stress. Eukaryotic Hsp70s conventionally have three domains—an N-terminal ~45 kDa ATPase domain, a substrate binding domain of ~15 kDa, and a C-terminal domain of 15 kDa. This C-terminal domain has a terminal sequence motif of EEVD, which has been implicated in binding proteins such as Hop (Hsp70 Hsp90 organizing protein) through their tetratricopeptide

☆ *Abbreviations:* Hsp70, heat shock protein; Hip, Hsp70 interacting protein; PfHsp70, heat shock protein from *Plasmodium falciparum*; PfHsp70-1, heat shock protein from *Plasmodium falciparum* coded by the gene annotated in PlasmodB as PF08_0054; PfHsp70-2, heat shock protein from *Plasmodium falciparum* coded by the gene annotated in PlasmodB as PF11_0351; PfHip, Hip from *Plasmodium falciparum*; Hsp90, heat shock protein 90; DSG, 15-deoxyspergualin; PfFabI, Enoyl acyl carrier protein reductase from *Plasmodium falciparum*; IPTG, isopropyl thio β-D-galactoside.

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motifs. The protein folding activity of heat shock protein 70 is brought about by successive binding and release events of nascent or unfolded protein substrates which are modulated by ATP binding, hydrolysis, and exchange. The ATPase activity of Hsp70 is stimulated by the Hsp70 interacting protein or Hip—a protein that is instrumental in stabilizing the assembly of Hsp70 and its substrate protein. Hip binds to the amino terminal ATPase domain of Hsp70 in an ADP dependent fashion through its centrally located tetratricopeptide repeat region and an adjoining highly charged region. The C-terminal GGMP tandem repeats of Hip may also bind concomitantly with the substrate-binding region of Hsp70, and the extreme N-terminal region is required for homo oligomerization [5].

Malaria, the dreaded disease caused by species of *Plasmodium*, remains a major cause of human mortality and morbidity especially in the tropics and subtropics of the world. Several heat shock proteins have been annotated in the *Plasmodium* database, PlasmodB, and genes coding for heat shock proteins in *Plasmodium falciparum* have been demonstrated to have unique G-rich regulatory elements [6]. The endogenous expression of various heat shock proteins has been studied in the malaria parasite, and they have been found to be thermoinducible [7–9]. The various temperatures that the malaria parasite encounters during its complex life cycle suggests that heat shock proteins should be indispensable for its survival. In fact, Hsp90 has been demonstrated to be essential for parasite survival, and geldanamycin, an inhibitor of Hsp90, abrogates parasite growth [10,11]. Several Hsp40 proteins from *P. falciparum* have been cloned [12], and a heat shock protein 70 has been cloned, overexpressed, and the recombinant protein demonstrated to suppress the thermosensitivity of an *Escherichia coli* DnaK mutant strain [13,14].

We report here the cloning, overexpression, purification, and preliminary characterization of three proteins of the heat shock family from *P. falciparum*—PfHsp70-1—an Hsp70 protein with a C-terminal EEVD motif encoded by the gene annotated in PlasmodB as PF08_0054, PfHsp70-2—an Hsp70 protein without a C-terminal "EEVD" motif annotated in PlasmodB as PF11_0351, and an Hsp70 interacting protein—PfHip annotated in PlasmodB as PFE1370w. More importantly, we report the effect of 15-deoxyspergualin (DSG), an Hsp70 binding molecule with antimalarial activity [15–18], on the chaperone activity of the recombinant heat shock proteins *in vitro*.

Experimental procedures

Materials. Malaria parasite culture components were obtained from Sigma. Primers for cloning were custom synthesized from Microsynth. *E. coli* strains DH5α and BL21-DE3, and expression vector, pET-28a(+), were obtained from Novagen. The pGEM-T easy kit was from Promega. The substrates and components for the chaperone assay, glutamate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase, and BSA were obtained from Sigma. Recombinant cloned PfFabI was expressed in BL21-DE3 cells, purified on His-bind resin, and used in the assay (see [19]). Anti-6× His antibody-horseradish peroxidase conjugate was obtained from Sigma.

Isolation of RNA from Plasmodium falciparum. The FCK2 parasite strain was cultured in O+ human red blood cells in RPMI medium supplemented with human serum by the candle jar method of Trager and Jensen [20]. Cultures were synchronized by 5% sorbitol treatment [21], and parasite growth was monitored by standard Giemsa staining. Total RNA was isolated from 10 ml of *P. falciparum* infected erythrocytes using an RNA isolation kit from AuPrep.

Cloning of PfHsp70-1. The primers used to amplify the 2034 bp gene from *P. falciparum* cDNA were designed based on the annotated sequence of PF08_0054 in PlasmodB (forward primer with *Bam*HI site: 5′-CgggATCCATggCTAgTgCAAAAggTTCAAAACC-3′, reverse primer with *Xho*I site: 5′-CCgCTCgAgTTAATCAACTTCTTCAACTgTTgg TCCAC-3′). The reverse transcriptase reaction was performed on the RNA using the reverse primer and a one-step kit from Qiagen. The PCR conditions used were 95 °C, initial denaturation for 5 min, 25 cycles of (94 °C, 1 min; 65 °C, 1 min; 72 °C, 2 min) and final extension at 72 °C, 5 min. The amplified PCR product was excised from a 0.8% agarose gel, purified using a silica gel column (Qiagen), and ligated into pGEM-T easy vector (Promega). The ligation mix was transformed into *E. coli* DH5α cells and candidate clones containing the correct-sized insert confirmed by sequencing. The PfHsp70-1 encoding *Bam*HI and *Xho*I fragment was restricted and ligated downstream of the 6X-His tag coding sequence of the *Bam*HI–*Xho*I restricted pET-28a(+) expression vector (Novagen). Restriction digestions using *Bam*HI and *Xho*I as well as DNA sequencing were used to identify and confirm the PfHsp70-1-pET-28a(+) construct.

Cloning of PfHsp70-2. Primers were designed for the 1992 bp annotated sequence of PF11_0351 from PlasmodB. The reverse transcriptase reaction was performed using a one-step kit from Qiagen using the designed reverse primer, and PCR (initial denaturation at 94 °C, 5 min, 25 cycles of 94 °C, 1 min; 65 °C, 1 min; 72 °C, 2 min; and final extension at 72 °C, 5 min) was carried out with the following primers:—forward (with *Nde*I site): 5′-ggAATTCCATATggCATCACTCAATAAAAAgAACATT gTAAAAATCC-3′ and reverse (with *Hin*dIII site) 5′-CCCAAgCTTTTA TgCATTATCTTTATTTTCTTCAgCTTTATTTTC-3′. The amplified PCR product was excised from a 0.8% agarose gel, purified using a silica gel column (Qiagen), and ligated into pGEM-T easy vector (Promega). The ligation mix was transformed into *E. coli* DH5α cells and candidate clones containing the correct-sized insert confirmed by sequencing. The PfHsp70-2 encoding *Nde*I and *Hin*dIII fragment was restricted and ligated downstream of the 6X-His tag coding sequence of the *Nde*I–*Hin*dIII restricted pET-28a(+) expression vector (Novagen). Restriction digestions using *Nde*I and *Hin*dIII as well as DNA sequencing were used to identify and confirm the PfHsp70-2-pET-28a(+) construct.

Cloning of PfHip. The primers used to amplify the 1377 bp gene from *P. falciparum* cDNA were designed based on the annotated sequence of PFE1370w in PlasmodB (forward primer with *Nde*I site: 5′-ggAATTC CATATgATAgATgAgAAAAAAgTTgAAgACTTAAAAC–3′, and reverse primer with *Bam*HI site: 5′-CgggATCCTTAATTTTTAC CATTCTCTTTTCCCATCATATTACC-3′). The reverse transcriptase reaction was performed on the RNA using the reverse primer and a one-step kit from Qiagen. The PCR conditions used were 94 °C, initial denaturation for 5 min, 25 cycles of (94 °C, 1 min; 65 °C, 1 min; 72 °C, 2 min), and final extension at 72 °C, 5 min. The amplified PCR product was excised from a 0.8% agarose gel, purified using a silica gel column (Qiagen), and ligated into pGEM-T easy vector (Promega). The ligation mix was transformed into *E. coli* DH5α cells and candidate clones containing the correct-sized insert confirmed by sequencing. The PfHip encoding *Nde*I and *Bam*HI fragment was restricted and ligated downstream of the 6X-His tag coding sequence of the *Nde*I–*Bam*HI restricted pET-28a(+) expression vector (Novagen). Restriction digestions using *Nde*I and *Bam*HI as well as DNA sequencing were used to identify and confirm the PfHip-pET-28a(+) construct.

Expression of recombinant PfHsp70-2, PfHsp70-1 and PfHip for protein purification. Overnight cultures of BL21-DE3 cells transformed with the respective expression plasmids were diluted into fresh LB medium containing 30 mg/ml kanamycin and grown with vigorous shaking at 37 °C to