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Isolation and characterization of *Plasmodium falciparum* UAP56 homolog: Evidence for the coupling of RNA binding and splicing activity by site-directed mutations ☆

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ABSTRACT

UAP56 (U2AF65 associated protein) is a member of the DEAD-box helicase family. Helicases are essential enzymes generally involved in the metabolism of nucleic acids. The gene encoding a member of DEAD-box family was cloned and characterized from the human malaria parasite *Plasmodium falciparum*. PfU52 is homologous to UAP56 and contains the RNA-dependent ATPase, RNA helicase and RNA binding activities. Using the parasite extract we report that PfU52 is involved in splicing reaction. Site-directed mutagenesis studies indicate that the conserved residues glycine 181, isoleucine 182 and arginine 206 are involved in RNA binding and this activity is required for the enzymatic activities of PfU52. PfU52 is expressed in all the intraerythrocytic developmental stages of the parasite. In the present study we have reported the detailed characterization of PfU52 from *P. falciparum* and these results advance the knowledge regarding the function of UAP56 in general.

Helicases in general use the free energies of ATP binding and hydrolysis to catalyze the unwinding of duplex nucleic acids and play an essential role in many cellular processes. The unwinding action by a helicase for the dissociation of duplex DNA strands into single-strand templates is a necessary step for the cellular processes of DNA replication, transcription and repair and each of these helicase-centered processes is crucial for the survival and propagation of the cells. The RNA helicases are generally involved in all the RNA-dependent cellular processes [1–3]. A number of helicases contain nine to eleven conserved amino acid sequence motifs, which are contained within a core element, flanked by specific amino and carboxy-terminal sequences [4,5]. These specific flanking sequences are assumed to interact with accessory proteins and impart specificity to these helicases [6]. The DEAD (Asp-Glu-Ala-Asp) motif, also known as the Walker B motif, is responsible for the name given to this family of proteins and this family belongs to the superfamily 2 of the helicase superfamilies [7]. Some of the conserved motifs are involved in ATP binding and hydrolysis while others are involved in RNA binding [4,5,8]. DEAD-box proteins are highly conserved from bacteria to mammals and are required for a variety of RNA metabolic processes such as transcription, ribosome biogenesis, splicing, RNA editing, RNA export from the nucleus, translation initiation and RNA turnover [6]. Only a few DEAD-box proteins are present in prokaryotic genomes but eukaryotes encode a number of these proteins, which are essential for survival or development of the organism [6,9]. UAP56/Sub2p (U2AF65 associated protein) is a member of the DEAD-box family of RNA helicases and has been shown to play important roles in the nuclear export of mature mRNA [10]. The UAP56/Sub2p proteins from different species contain a DECD motif in place of the common DEAD motif and the mammalian UAP56 was initially reported as a U2AF65 interacting protein [10,11].

The most dangerous of the four species of genus *Plasmodium* that cause human malaria is the malaria parasite *Plasmodium falciparum* [12]. It causes the most problematic disease in humans as a result of its prevalence, virulence and drug resistance [13]. The pathogenicity of this parasite mainly results from its rapid rate of asexual reproduction in the host and its ability to sequester in small blood vessels [12]. In the continuing absence of clinically proven vaccines, the treatment of malaria parasite infections in the human host mainly depends on chemotherapy [13]. But due to the lack of new affordable drugs there are only a limited number of drugs in widespread use for the treatment of *P. falciparum* malaria and it has developed resistance to nearly all the available anti-malarial drugs [13,14]. The search for novel effective, safe and affordable anti-malarial drugs for *P. falciparum* malaria is one of the most important tasks to pursue and thus there is a need for new drugs and the identification of new chemotherapeutic targets. In the case of *P. falciparum,* based on differential gene expression study in the presence of chloroquine, it has been predicted that RNA helicase like proteins may be involved in anti-malarial action of the drug and recently it has been reported that helicases could be feasible anti-malarial drug targets [15,16]. We have started a systematic study of helicases and it was reported that the *P. falciparum* genome contains a number of DEAD-box helicases [17,18]. Recently we have reported the characterization of one of

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the proteins of DEAD-box family from *P. falciparum* [19,20]. In the present study we report the cloning and detailed characterization of a 52 kDa homolog of UAP56 designated as PfU52 from *P. falciparum*. PfU52 is an RNA helicase with intrinsic RNA-dependent ATPase activity and it is expressed in all the asexual stages of the development of the parasite. The site-directed mutagenesis studies indicate that the amino acids glycine 181, isoleucine 182 and arginine 206 of PfU52 are essentially required for the RNA binding, RNA-dependent ATPase and splicing activities of PfU52. This study demonstrates the characterization of a novel functionally active RNA helicase from *P. falciparum*.

Materials and methods

Isolation of RNA and preparation of cDNA

Plasmodium falciparum (strain 3D7) was cultured as described [21]. Total RNA was isolated and was used for the preparation of cDNA using a cDNA synthesis kit (Superscript first-strand synthesis system from Invitrogen, Carlsbad, CA, USA). The *P. falciparum* helicase cDNA was amplified using the forward primer Pf1F, 5′-*GGATC*-CATGGCTTCAATGGATCATAATGC-3′ and the reverse primer Pf1R, 5′-*AAAGCTT*TTATTGATTGATGTACTCATT-3′. The restriction sites are written in italics.

PCR condition for amplification was initial denaturation—95° C for 5 min; 95° C for 1 min, 50° C with increment of 0.5° C every cycle, 72° C for 1 min for 30 cycles; 72° C for 2 min-final extension. As a template cDNA was used since the genomic DNA contains one intron. The PCR product of ~1.4 kb was gel purified and cloned into the pGEM-T vector from Promega (Madison, WI, USA) and the clone was sequenced by automated DNA sequencing (Macrogen, Korea). The nucleotide sequence was submitted in GenBank and the Accession No. is EF690547. The DNA band was excised using BamHI and HindIII enzymes (New England Biolabs, Beverly, MA, USA) and gel purified for subcloning into the expression vector.

Expression and purification of the protein

The complete open reading frame of PfU52 was subcloned into the expression vector pET28a (Novagen, Madison, WI, USA) at BamHI and HindIII sites. The expression clones were transformed into *Escherichia coli* strain BL21 (DE3) pLysS and the expressed protein was purified using standard methods with Ni-NTA (Qiagen, GmbH, Germany) affinity chromatography. The recombinant his-tagged protein was eluted with 50 mM imidazole in protein buffer and was checked by SDS–PAGE analysis. The purity of the protein was checked by silver staining using the standard protocol [22].

ssDNA-dependent and RNA-dependent ATPase assays

The hydrolysis of ATP catalyzed by PfU52 and various mutants was assayed by measuring the formation of inorganic phosphate (Pi) from [γ-^{32}P] ATP. The reaction was performed for 2 h at 37° C both in the presence and absence of 100 ng of M13 mp19 ssDNA and RNA from *P. falciparum*, followed by thin-layer chromatography and quantification as described [23].

Antibody production and Western blot analysis

Purified PfU52 was used for the production of polyclonal antibodies in mice using standard protocols [24]. The polyclonal antibodies were purified as IgG fractions using protein-A Sepharose as described earlier [24]. For Western blot analysis, the proteins were separated by SDS–PAGE and transferred electrophoretically to nitrocellulose membrane as described [24]. After blocking, the membrane was incubated with the appropriate primary (Penta-His from Qiagen, GmbH, Germany) or specific antibody for 3 h at room temperature. After washing, the blot was incubated with the appropriate secondary antibody coupled to alkaline phosphatase from Sigma (St. Louis, MO, USA) and developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium obtained from Sigma (St. Louis, MO, USA).

For Western blot analysis of parasite proteins, the parasites were synchronized using sorbitol and harvested at various time-points. Total proteins (100 μg) from the lysate representing various intraerythrocytic developmental stages were separated by SDS–PAGE and the proteins were transferred to nitrocellulose membrane as described [24]. The blot was probed with 1:1000 dilutions of purified PfU52 antisera raised in mice and developed using appropriate secondary antibody as described above.

Bioinformatics analysis and construction of mutants

Different softwares were used to map the RNA binding amino acid residues of the protein PfU52. The softwares available on http://bindr.gdcb.iastate.edu/RNABindR, http://yayoi.kansai.jaea.go.jp/qbg/kyg/ and http://jing.cz3.nus.edu.sg/cgi-bin/svmprot.cgi were used. Each of the residues having high propensity were replaced once with alanine and again mapped using the above software (Table 1). The substitution(s) which gave maximum change in RNA binding propensity of the full length protein were selected for mutation analysis and various mutants were constructed. The site-directed mutagenesis was carried out by PCR using QuickChange™ site-directed mutagenesis Stratagene kit (La Jolla, CA, USA) as recommended by the manufacturer. The pGEM-T plasmid harboring *PfU52* was used as DNA template. The primers synthesized from Sigma and used for the mutagenesis are summarized in Table 2. The mutants were confirmed by DNA sequencing and the mutant proteins were purified using the same procedure as described for PfU52.

Circular dichroism spectroscopy measurements

Circular dichroism (CD) measurements were performed with a Jasco J-810 spectropolarimeter. The samples were analyzed in quartz cells with path lengths of 1 mm. Near-UV wavelength scans were recorded from 190 to 250 nm. All the CD spectra were corrected by subtraction of the background for the spectrum obtained with either buffer alone. The average of three wavelength scans is presented. The ellipticity results were expressed as mean residue ellipticity, [θ], in degrees cm^2/dmol.

In vitro RNA binding assay

The RNA binding assay was done by using the method described previously [25]. For this equal amounts (1 μg) of BSA, PfU52 (full length) and mutant proteins M1 to M4 were dot-blotted on pre-charged PVDF membrane. This membrane was blocked for 1 h at room temperature in a blocking buffer (25 mM NaCl, 10 mM $MgCl_2$, 10 mM HEPES, 0.1 mM EDTA, 1 mM DTT, 3% BSA). 13 mer RNA oligo (5′-AUAGCCUCAACCG-3′) synthesized from Primm srl (Milan, Italy) was labeled at the 5′-end with 1.85 MBq of [γ-^{32}P] ATP (specific activity 222 TBq/mmol) using T4 polynucleotide kinase (NEB, England) and purified by Sepharose 4B column (Pharmacia, Sweden). The membrane after 1 h of blocking was further incubated for 2 h in a binding buffer (50 mM NaCl, 10 mM $MgCl_2$, 10 mM HEPES, 0.1 mM EDTA, 1 mM DTT, 1.5% BSA) containing 10 pmol of ^{32}P-labeled RNA substrate. After binding, the membrane was washed thrice with binding buffer and exposed for auto-radiography. The spots obtained were quantitated by densitometry.