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Plasmodium falciparum DOZI, an RNA helicase interacts with eIF4E

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ABSTRACT

DEAD box RNA helicases play crucial roles in RNA metabolism such as splicing, ribosome biogenesis, RNA transport, degradation and translation. DDX6/DOZI (development of zygote inhibited) is one of the well characterized member of the DEAD box family and is highly conserved from humans to malaria parasite. DDX6 is involved in a variety of biological processes, which include the sexual development of the protozoan parasite. In the present manuscript we report that *P. falciparum* DOZI (DDX6 homologue); PfDZ50 contains the characteristic DNA and RNA binding, nucleic acid-dependent ATPase and RNA unwinding activities. Enzymatic characterization of truncated derivatives of PfDZ50 such as PfDZ50T1 (domain 1) and PfDZ50T2 (domain 2) shows that none of them contains ATPase activity. Furthermore, we confirmed that PfDZ50 interacts with PfeIF4E mainly through domain 1. Using in vitro translation assays we show that PfDZ50 inhibits translation. With the same assays we further report that externally added PfeIF4E restores ~70% of translation. Using immunofluorescence assays we demonstrate that PfDZ50 is localized mainly in the cytoplasm in the asexual intraerythrocytic developmental stages of *P. falciparum*. The localization pattern further suggests that PfDZ50 appears typically in granular bodies throughout the cytoplasm. Thus these studies will advance our knowledge regarding the function of PfDZ50/DDX6 in general.

1. Introduction

The ability to unwind double stranded RNAs or DNA duplexes has been attributed to specific enzymes commonly known as helicases or unwindases. These enzymes work in an energy-dependent manner through the hydrolysis of nucleoside triphosphates (NTPs). Helicases are ubiquitous and present in almost all organisms ranging from bacteria and viruses, malaria parasite to humans (Tuteja, 2010; Umate et al., 2011). These enzymes are members of the well-characterized DEAD-box family, which was named according to one of its conserved motifs (Linder et al., 1988; Wassarman and Steitz, 1991). The sequence analysis of RNA helicases revealed that they also contain almost similar conserved motifs which are present in most of the DNA helicases (Gorbalenya and Koonin, 1993). It is well established that the DEAD-box helicases are involved in almost all the metabolic processes involving RNA such as splicing, ribosome biogenesis, RNA transport, degradation and translation. Helicases mostly use NTP (usually ATP) binding and hydrolysis to remodel RNA, or RNA–protein complexes and this action results in double-stranded RNA unwinding, and/or displacement of proteins from RNA (Cordin et al., 2006; Rocak and Linder, 2004). The catalytic core of helicases is generally composed of two RecA-like domains with nine conserved domains, including the DEAD-box motif and the Q motif, which has roles in catalysis and substrate binding. The DEAD-box p54 RNA helicase is a member of a superfamily 2/DDX6 helicase subfamily and is highly conserved from trypanosomes, malaria parasite to humans with well-characterized homologues in *Xenopus laevis* (Xp54), *Drosophila melanogaster* (Me31B), *Caenorhabditis elegans* (CGH-1), and *Saccharomyces cerevisiae* (Dhh1) (Mair et al., 2006; Rajyaguru and Parker, 2009; Tuteja, 2010). The DEAD-box p54 RNA helicase family is significant due to its involvement in a variety of biological processes, which include the sexual development of the protozoan *Plasmodium*, the regulation of multiple virulence-associated genes in *Cryptococcus neoformans*, germline apoptosis, and embryonic cytokinesis regulation in *C. elegans* (Beckham and Parker, 2008; Mair et al., 2006; Rajyaguru and Parker, 2009). It has been reported in previous studies that DDX6-like proteins can apparently interact with a range of different protein partners in a variety of cellular situations (Weston and Sommerville, 2006). DDX6 is localized in the cytoplasm and thus plays a role at the protein translational level and/or mRNA stability (Abdelhaleem, 2004). It has been reported earlier that the *Xenopus* homologue Xp54 is an integral component of messenger ribonucleoprotein (mRNP) particles and it acts as a factor involved in translational control (Ladomery et al., 1997). The studies reported thus far indicate

Abbreviations: ATP, adenosine tri-phosphate; ATPase, adenosine tri-phosphatase; DAB, 3,3′-diaminobenzidine; DAPI, 40,60-di-amidino-2-phenylindole dihydrochloride; DOZI, development of zygote inhibited; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; NTP, nucleoside triphosphate; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PfeIF4E, *Plasmodium falciparum* eukaryotic initiation factor 4E; ss-DNA, single stranded DNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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that this subfamily of helicases serves as a key component in the metabolism of mRNA (Weston and Sommerville, 2006).

It is well known that *Plasmodium* is haploid throughout its life cycle and the sexual development of the malaria parasite commences with the generation of gametocytes in the human host (Tuteja, 2007). In an interesting study in *Plasmodium berghei* it has been reported that the translationally dormant mRNAs are found in the cytoplasm of female gametocytes and their stabilization and maintenance depends on this DEAD-box RNA helicase termed DOZI (development of zygote inhibited) (Mair et al., 2006) which is a homologue of DDX6. It was further reported that in the absence of DOZI, gametocytes undergo large scale mRNA destabilization which results in the abortion of ookinete development soon after zygote formation (Mair et al., 2006). In a related study, a messenger ribonucleoprotein (mRNP) from *P. berghei* gametocytes defined by DOZI and the Sm-like factor CITH (homolog of worm CAR-I and fly Trailer Hitch) was identified (Mair et al., 2010). Their analysis indicated that mRNP includes 16 common major protein factors including eIF4E and poly(A)-binding protein (Mair et al., 2010).

In the present study we report the detailed biochemical characterization of the DOZI homologue commonly known as DDX6 from *Plasmodium falciparum* 3D7 strain. *P. falciparum* DOZI homologue, PfDZ50 contains the characteristic nucleic acid-dependent ATPase, RNA and DNA binding and RNA unwinding activities but it has no detectable DNA unwinding activity. The characterization of truncated derivatives of PfDZ50 such as PfDZ50T1 (domain 1) and PfDZ50T2 (domain 2) shows that none of them contains the enzymatic activity and full-length PfDZ50 is required for the ATPase activity. Using ELISA-based interaction assays we show that PfDZ50 interacts with PfeIF4E. PfDZ50 interacts with PfeIF4E mainly through domain 1 because when the truncated derivatives, PfDZ50T1 (domain 1) and PfDZ50T2 (domain 2) were used, it was observed that the interaction with PfDZ50T2 is almost negligible as compared to PfDZ50T1, which showed almost 50% interaction in comparison to PfDZ50. Using in vitro translation assays we further show that PfDZ50 inhibits translation. The supplementation of the lysate with externally added recombinant PfeIF4E restores ~70% of the translation. It is interesting to note that PfDZ50 (PfDDX6) is expressed in all the intraerythrocytic developmental stages i.e. ring, trophozoite and schizont stages in the parasite *P. falciparum* 3D7 strain. Using immunofluorescence assays we demonstrate that PfDDX6 is localized mainly in the cytoplasm in the *P. falciparum* 3D7 strain. To the best of our knowledge this is the first study reporting the direct interaction between PfDZ50 and PfeIF4E and these studies will advance our knowledge regarding the function of DDX6 in general.

2. Materials and methods

2.1. Identification and cloning of P. falciparum PfDZ50gene

The *P. falciparum* helicase gene was amplified using the forward primer PfDZ50F1 with BamHI site and the reverse primer PfDZ50R1 with HindIII site using *P. falciparum* 3D7 cDNA as template. The PCR conditions used were 95 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min. This was repeated for a total of 35 cycles and at the end one elongation was done at 72 °C for 12 min. The PCR product of ~1.3 kb was gel purified and cloned into the pGEM-T easy vector from Promega (Madison, WI, USA) and the clones were sequenced by dideoxy sequencing reactions (Macrogen, Korea). The nucleotide sequence was submitted to GenBank and the accession no. is EF070216.1. For the amplification of domain 1 (from amino acid 1–254) separately, PfDZ50F1 with BamHI site and PfDZ50R2 with HindIII site were used. For the amplification of domain 2 (from amino acid 255–433) separately, PfDZ50F2 with BamHI site and PfDZ50R1 with HindIII site were used and the PCR conditions used were 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min.

1. PfDZ50F1, 5′-GGGATCCATGAGTTATAAAACCAATT-3′ (BamHI site)
2. PfDZ50R1, 5′-CAAGCTTTTAGGTATATAAGGATGGGT-3′ (HindIII site)
3. PfDZ50F2, 5′-GGGATCCTTATCAGATGCCCATGAAATAAATC-3′ (BamHI site)
4. PfDZ50R2,5′-CAAGCTTGATTTATTTCATGGGCATCTGATAA-3′ (HindIII site)

The DNA bands were excised using BamHI and HindIII enzymes (New England Biolabs, Beverly, MA, USA) and gel purified for subcloning into the expression vector pET28a.

2.2. Expression and purification of recombinant protein in Escherichia coli

The fragment excised from pGEMT easy vector was stitched in the pET28a (Novagen, Madison, WI, USA) at the BamHI and HindIII sites. The expression clones were transformed into *E. coli* strain BL21 (DE3) pLysS and the expression of recombinant protein was induced by 1 mM IPTG. The expressed protein was purified using standard methods with Ni-NTA (Qiagen, GmbH, Germany) affinity chromatography. The recombinant his-tagged protein was eluted with 200 mM imidazole in protein buffer (20 mM Tris–HCl (pH 8.0), 250 mM NaCl, 10% (v/v) glycerol and protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA) and was checked for purity by SDS-PAGE (10% (w/v) polyacrylamide gel) and silver staining using slight modifications of the standard protocol (Sambrook et al., 1989). The slight modification included extensive washing of the gel after fixation and this step reduces the background and increases the sensitivity of the stain.

2.3. Generation of polyclonal antisera

Purified PfDZ50 was used for the preparation of antibodies in mice using the standard protocols (Sambrook et al., 1989). The polyclonal antibodies were purified as IgG fractions using protein A-Sepharose as described (Sambrook et al., 1989).

2.4. Preparation of DNA helicase substrate

The helicase activity of PfDZ50 was determined by the standard strand displacement assay using the partially duplex substrate and the method described previously (Pradhan and Tuteja, 2007). Ten nanograms of the oligodeoxynucleotide was labeled at 5′-end with T4 polynucleotide kinase (5 U) and 1.85 MBq of [γ-^{32}P]ATP (specific activity 222 TBq/mmol) in standard conditions. The labeled oligodeoxynucleotide was then annealed with 0.5 μg of single-stranded circular M13mp19 (+) DNA using standard annealing buffer by heating at 95 °C for 1 min, transferring immediately to 65 °C for 2 min and then cooling slowly to room temperature. The non-hybridized oligodeoxynucleotide was removed using gel filtration through a Sepharose 4B column (Pharmacia, Sweden). The reaction mixture (10 μl) containing appropriate buffer, the ^{32}P-labeled helicase substrate (1000 cpm/10 μl) and the purified protein fraction to be assayed was incubated at 37 °C for 60 min. The substrate and products were separated by electrophoresis on a nondenaturing 12% PAGE (Tuteja et al., 1993) and the gel was scanned on phosphoimager and both the substrate and unwound DNA bands were quantified.

2.5. ATPase assay

The hydrolysis of ATP catalyzed by PfDZ50 was assayed by measuring the formation of ^{32}P from [γ-^{32}P] ATP. The reaction conditions were identical to those described for the helicase reaction, except that the ^{32}P-labeled helicase substrate was replaced by a mixture of [γ-^{32}P] ATP (specific activity 222 TBq/mmol^{-1}) and cold ATP (1 mM). The reaction was performed for 2 h at 37 °C (or one hour)