International Journal for Parasitology 41 (2011) 417–427

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Interaction of apicoplast-encoded elongation factor (EF) EF-Tu with nuclear-encoded EF-Ts mediates translation in the *Plasmodium falciparum* plastid

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ARTICLE INFO

Article history:
Received 28 September 2010
Received in revised form 8 November 2010
Accepted 8 November 2010
Available online 14 December 2010

Keywords:
Plasmodium falciparum
Apicoplast
Translation
EF-Tu
EF-Ts
Kirromycin

ABSTRACT

Protein translation in the plastid (apicoplast) of *Plasmodium* spp. is of immense interest as a target for potential anti-malarial drugs. However, the molecular data on apicoplast translation needed for optimisation and development of novel inhibitors is lacking. We report characterisation of two key translation elongation factors in *Plasmodium falciparum*, apicoplast-encoded elongation factor *Pf*EF-Tu and nuclear-encoded *Pf*EF-Ts. Recombinant *Pf*EF-Tu hydrolysed GTP and interacted with its presumed nuclear-encoded partner *Pf*EF-Ts. The EF-Tu inhibitor kirromycin affected *Pf*EF-Tu activity in vitro, indicating that apicoplast EF-Tu is indeed the target of this drug. The predicted *Pf*EF-Ts leader sequence targeted GFP to the apicoplast, confirming that *Pf*EF-Ts functions in this organelle. Recombinant *Pf*EF-Ts mediated nucleotide exchange on *Pf*EF-Tu and homology modeling of the *Pf*EF-Tu:*Pf*EF-Ts complex revealed *Pf*EF-Ts-induced structural alterations that would expedite GDP release from *Pf*EF-Tu. Our results establish functional interaction between two apicoplast translation factors encoded by genes residing in different cellular compartments and highlight the significance of their sequence/structural differences from bacterial elongation factors in relation to inhibitor activity. These data provide an experimental system to study the effects of novel inhibitors targeting *Pf*EF-Tu and *Pf*EF-Tu.*Pf*EF-Ts interaction. Our finding that apicoplast EF-Tu possesses chaperone-related disulphide reductase activity also provides a rationale for retention of the *tufA* gene on the plastid genome.

1. Introduction

The malaria parasite *Plasmodium falciparum* and related apicomplexans contain a relict plastid called the apicoplast. The apicoplast is essential for parasite survival (Fichera and Roos, 1997; McFadden and Roos, 1999) and several biochemical pathways appear to function within the organelle (Ralph et al., 2004). In addition to these pathways, the housekeeping processes of apicoplast DNA replication, transcription and translation have been recognised as sites for drug intervention (Ralph et al., 2001; Foth and McFadden, 2003). Although some such inhibitors cause a delayed-death effect, the irreversible lethal effects of loss of plastid function nevertheless makes some of them plausible chemotherapeutic agents. In particular, there is interest in the use of known and novel prokaryotic translation inhibitors that specifically target the apicoplast in anti-malarial therapy (Goodman et al., 2007; Dahl and Rosenthal, 2008). Indeed, two such inhibitors, doxycycline and clindamycin, are already in use; the former is recommended as a malaria prophylactic for travellers to endemic areas while the latter is in advanced human clinical trials in combination with another drug with apicoplast-specific action (Borrmann et al., 2006; Ruangweerayut et al., 2008).

The evidence for active translation in the *P. falciparum* apicoplast is compelling. The 35 kb circular DNA genome (plDNA) of the organelle carries genes encoding 16S and 23S rRNAs, 25 tRNAs, translation elongation factor Tu (*tufA*) and 29 other proteins, including 18 ribosomal proteins (Wilson et al., 1996). As the complement of apicoplast-encoded ribosomal proteins is insufficient for 70S ribosomal assembly, it is believed that a large component of ribosomal proteins is nuclear-encoded and imported by the apicoplast (Wilson et al., 1996). A number of these have been identified to contain the bipartite import signal characteristic of apicoplast-targeted proteins (Foth et al., 2003) and apicoplast localisation of the nuclear-encoded ribosomal proteins S9 and L28 has been demonstrated in the related apicomplexan *Toxoplasma gondii* (Waller et al., 1998). 70S

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0020-7519/$36.00
doi:10.1016/j.ijpara.2010.11.003

ribosome-like particles in the apicoplast are apparent by electron microscopy (McFadden et al., 1996). Ribosome-like particles that carry plastid-specific mRNA and rRNAs have also been detected (Roy et al., 1999). Conclusive evidence for active translation in the organelle has come from the detection of the *tufA* gene product in the apicoplast and the inhibitory effect of the prokaryotic translation inhibitor thiostrepton on elongation factor Tu (EF-Tu) levels (Chaubey et al., 2005). Clindamycin, an inhibitor that binds bacterial lsrRNA, inhibits *Plasmodium* growth, and a single point mutation in the lsrRNA gene of the *T. gondii* apicoplast confers clindamycin resistance in vitro (Camps et al., 2002). Azithromycin resistance generated in parasite lines has been attributed to a point mutation in the *P. falciparum* apicoplast lsrRNA gene as well as the gene encoding the apicoplast-encoded ribosomal protein subunit Rpl4 (Sidhu et al., 2007). In addition, apicoplast-specific effects such as disruption of protein import into the organelle by clindamycin and tetracycline (Goodman et al., 2007) have been observed. Doxycycline apparently blocks expression of the apicoplast genome resulting in the distribution of non-functional apicoplasts during erythrocytic schizogony (Dahl et al., 2006a). A recent study (Stanway et al., 2009) using live microscopy on *Plasmodium berghei* has shown that microbial translation inhibitors also block development of the apicoplast during exo-erythrocytic schizogony in liver stages, leading to impaired parasite maturation.

Despite strong interest in the process of apicoplast translation as a target for anti-malarial drugs, there is minimal information on functional and structural aspects of initiation, and elongation factors participating in the process. Although they remain to be functionally characterised, translation factors that may participate in protein synthesis in the apicoplast are listed in Supplementary Table S1. During protein synthesis in prokaryotes, amino acyl-tRNA (aa-tRNA) is delivered to the ribosomal A-site by EF-Tu which hydrolyses GTP and releases the tRNA after codon recognition (Schmeing et al., 2009). The second elongation factor, EF-Ts, binds to the structurally distinct EF-Tu.GDP form that is released from the ribosome and mediates GDP release, thus regenerating EF-Tu for another round of GTP binding and aa-tRNA delivery. For catalysis of the nucleotide exchange reaction, EF-Ts binds to EF-Tu.GDP and GDP is rapidly released from the unstable EF-Tu.GDP.EF-Ts complex. Subsequent binding of GTP forms the EF-Tu.GTP.EF-Ts complex which then dissociates into EF-Ts and the active EF-Tu.GTP form (Dahl et al., 2006b). EF-G binds the ribosome sequentially after EF-Tu and activates translocation, the movement of deacylated-tRNA from the ribosome P-site to the E-site and concurrent transfer of the peptidyl-tRNA from the A-site to the P-site, thus advancing the mRNA one codon in the 3′ direction. The A-site is thus freed for entry of another EFTu.GTP.aa-tRNA ternary complex (Yu et al., 2009). Of the three elongation factors required for apicoplast translation only one, EF-Tu, is encoded by the apicoplast genome. It is highly similar to other EF-Tu molecules, particularly to the orthologous functional domains (Wilson et al., 1996; Sato et al., 2000). No EF-Ts or EF-G are encoded by the apicoplast genome but the current PlasmoDB annotation identifies genes encoding EF-Ts (PFC0225c) and EF-G (PFF0115c) homologs on chromosomes 3 and 6 of *P. falciparum*, respectively, with an apparent bipartite apicoplast-targeting leader encoded at the 5′ end of each (Foth et al., 2003) (Supplementary Table S1).

Among the drugs that target EF-Tu is kirromycin, a complex linear polyketide. The antibiotic blocks protein synthesis by inhibiting the release of bacterial EF-Tu.GDP from the ribosome. The EF-Tu.GDP.kirromycin complex locks bacterial EF-Tu in an EFTu.GTP-like conformation that remains on the ribosome, thus preventing the peptidyl transferase reaction and subsequent translocation (Parmeggiani and Swart, 1985; Vogeley et al., 2001). Kirromycin also alters the behaviour of *Escherichia coli* EF-Tu such that the activities elicited by cellular effectors are mimicked (Mesters et al., 1994). Kirromycin is capable of enhancing GDP release from bacterial EF-Tu in the absence of EF-Ts and stimulates GTP hydrolysis in the absence of ribosome and aa-tRNA (Wolf et al., 1974; Mesters et al., 1994). The drug has been shown to exhibit antimalarial activity in *P. falciparum* blood culture with an IC_{50} of ~50 μM and can bind to the *P. falciparum* apicoplast EF-Tu.GDP (Clough et al., 1999).

Attempts at functional characterisation of the *P. falciparum* apicoplast EF-Tu (*Pf*EF-Tu) and its interaction with potential inhibitors have been made in the past but have met with limited success due to difficulties associated with recombinant expression of the protein as a functionally active molecule (Clough et al., 1999; Chaubey et al., 2005). We describe the use of re-folded functional apicoplast EF-Tu, expressed in *E. coli* as a fusion protein with maltose binding protein (MBP), to study *P. falciparum* EF-Tu (*Pf*EF-Tu) function, its interaction with *Pf*EF-Ts as well as the effect of kirromycin on its activity. Our results establish apicoplast localisation of nuclear-encoded *Pf*EF-Ts and its ability to mediate nucleotide exchange on *Pf*EF-Tu. We believe this provides the first functional characterisation of two critical components of the translation machinery of the *Plasmodium* apicoplast.

2. Materials and methods

2.1. Parasite culture and cDNA preparation

Plasmodium falciparum (strain 3D7) was cultured as described by Jensen and Trager (1978). Parasite genomic DNA was isolated by phenol/chloroform extraction. Total parasite RNA was isolated with Trizol Reagent (Invitrogen) and reverse transcribed (RT) using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen).

2.2. Recombinant protein expression and purification

Primers were designed to PCR-amplify the nuclear DNA sequence encompassing the two exons encoding *Pf*EF-Ts (PlasmoDB ID PFC0225c) as well as only the first exon of the gene. The entire exon 1 was PCR-amplified using upstream (5′-CGCGGATCCAAA TTGTTTTACTTTTTCTTGT TAAGC-3′) and downstream (5′-CGCGTC GACCTGCTCTAGGAATACTATG-3′) primers carrying *Bam*HI and *Sal*I tags (underlined) with genomic DNA as template. The amplified DNA was cloned into pET23a vector with a polyhistidine (His)-tag at the N-terminus generating the construct pET-Ts_{exon1}. *E. coli* BL-21 DE3 cells were co-transformed with pET-Ts_{exon1} and the RIG plasmid (gift from Prof. W.G.J. Hol, Howard Hughes Medical Institute, USA). The protein was expressed as inclusion bodies and PAGE-separated, electroeluted protein (Ram et al., 2008) was used to generate antibodies against *Pf*EF-Ts in rabbit. Approval for animal use was given by the Institutional Animal Ethics Committee of the Central Drug Research Institute, India. Maintenance and care of animals was in accordance with Government of India guidelines.

The DNA sequence encoding processed EF-Ts (protein lacking the predicted signal-transit sequence for apicoplast targeting) was amplified using upstream (5′-CGCGGATCCGATCATCTAAAA CTATTAAAATATG-3′) and downstream (5′-CGCGTCGACTTCCATAA GAA CGTTTTTTTCCCC-3′) primers carrying *Bam*HI and *Sal*I tags. cDNA prepared from total parasite RNA was used as template. The amplified sequence was cloned into the pGEX-KG vector and the resultant pGEX-Ts_f vector and the RIG plasmid were co-transformed into *E. coli* XL-1 blue cells. The GST-*Pf*EFTs fusion protein (~65 kDa) with N-terminal GST was expressed in the soluble fraction and was purified on a glutathione Sepharose affinity column (GE Healthcare). For removal of the GST-tag, fusion protein bound to the affinity column was cleaved with thrombin. The eluted