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Dual targeting of aminoacyl-tRNA synthetases to the apicoplast and cytosol in *Plasmodium falciparum*

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ABSTRACT

The causative agent of malaria, *Plasmodium*, possesses three translationally active compartments: the cytosol, the mitochondrion and a relic plastid called the apicoplast. Aminoacyl-tRNA synthetases to charge tRNA are thus required for all three compartments. However, the *Plasmodium falciparum* genome encodes too few tRNA synthetases to supply a unique enzyme for each amino acid in all three compartments. We have investigated the subcellular localisation of three tRNA synthetases (AlaRS, GlyRS and ThrRS), which occur only once in the nuclear genome, and we show that each of these enzymes is dually localised to the *P. falciparum* cytosol and the apicoplast. No mitochondrial fraction is apparent for these three enzymes, which suggests that the *Plasmodium* mitochondrion lacks at least these three tRNA synthetases. The unique *Plasmodium* ThrRS is the presumed target of the antimalarial compound borrelidin. Borrelidin kills *P. falciparum* parasites quickly without the delayed death effect typical of apicoplast translation inhibitors and without an observable effect on apicoplast morphology. By contrast, mupirocin, an inhibitor of the apicoplast IleRS, kills with a delayed death effect that inhibits apicoplast growth and division. Because inhibition of dual targeted tRNA synthetases should arrest translation in all compartments of the parasite, these enzymes deserve further investigation as potential targets for antimalarial drug development.
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1. Introduction

Treatment of malaria is becoming increasingly complicated by the widespread resistance of *Plasmodium* parasites to many existing antimalarial drugs. This generates an intense and urgent need for development of new antimalarial drugs that are not compromised by existing resistance mechanisms. At least two antimalarial drugs, doxycycline and clindamycin, inhibit components of the protein translation machinery within the apicoplast (a relic plastid). The efficacy of these drugs validates protein translation as a key drug target in *Plasmodium*, and suggests other elements of the cytosolic or apicoplast protein translation machinery could act as potential targets for antimalarial compounds. One such family of enzymes involved in translation that has been advanced as promising drug targets is the aminoacyl-tRNA synthetase (aaRS) family. These enzymes are responsible for the charging of individual tRNA molecules with their cognate amino acids (Ibba and Soll, 2000; Ochsner et al., 2007). *Plasmodium*, like other eukaryotes, possesses organellar as well as cytosolic tRNA synthetases and several aaRS inhibitors inhibit growth of *Plasmodium* spp. The threonyl-tRNA synthetase inhibitor, borrelidin, potently kills *Plasmodium falciparum* parasites grown in culture (Ishiyama et al., 2011) and cures mice of rodent malaria infections (Otoguro et al., 2003). A clinically used inhibitor of bacterial type isoleucyl-tRNA synthetase, mupirocin (Ward and Campoli-Richards, 1986; Gurney and Thomas, 2011) and an inhibitor of the eukaryotic type isoleucyl-tRNA synthetase, thiaisoleucine, also inhibit growth of cultured *P. falciparum* (Istvan et al., 2011).

Inhibition of cytosolic protein machinery in *Plasmodium* generally leads to an immediate growth arrest, whereas inhibition of apicoplast-targeted protein machinery leads to a characteristic delayed death phenotype, where parasites do not die until the subsequent round of replication after treatment (Dahl and Rosenthal, 2007). Thiaisoleucine, an apparent inhibitor of the *Plasmodium* cytosolic IleRS, produced an immediate effect, whereas mupirocin,

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an apparent inhibitor of the *Plasmodium* apicoplast IleRS, produced parasite growth inhibition consistent with the delayed death phenotype (Istvan et al., 2011). The action of the ThrRS inhibitor, borrelidin, against *Plasmodium* is at face value unusual because borrelidin kills parasites rapidly as would be expected for a cytosolic target enzyme, but the single ThrRS encoded by the *P. falciparum* genome (PF11_0270) (Bhatt et al., 2009; Jackson et al., 2011), starts with a very strongly-predicted apicoplast trafficking leader sequence (Foth et al., 2003). We therefore investigated the trafficking of this enzyme to study the nature of inhibition by borrelidin.

A distinct aaRS is needed to charge each tRNA with its cognate amino acid. Eukaryotic organelles such as plastids and mitochondria that have active translation machineries also require aaRS enzymes. Organellar genomes often encode all of their own tRNA molecules but all known aaRSs are nuclear encoded, so organellar aaRSs must be synthesised in the cytosol and post-translationally trafficked to their destination (Duchene et al., 2009). In numerous cases the demand for aaRS enzymes in the cytosol and in an organelle is met by dually targeting the same nuclear-encoded aaRS to both locations. Thus, for many aaRSs in *Saccharomyces cerevisiae*, a single nuclear gene encodes a protein that is localised to the cytosol as well as the mitochondria (Natsoulis et al., 1986; Chatton et al., 1988). In *Arabidopsis* the situation is even more complex, with some aaRS gene products being dually targeted to the chloroplast and the mitochondrion, and even some aaRSs that are found in the cytosol and both endosymbiotic organelles (Souciet et al., 1999; Duchene et al., 2005). Differential solubilisation experiments indicate that some aaRSs in the apicomplexan *Toxoplasma gondii* are also dually targeted to multiple compartments (Pino et al., 2009). The mechanisms for many of these multiple targeting events remain unclear.

We searched for possible examples of multiple targeted aaRSs encoded by *P. falciparum* genes (Gardner et al., 2002). As noted previously (Bhatt et al., 2009; Pino et al., 2009; Jackson et al., 2011), several aaRS proteins are found only once in the *Plasmodium* genome, including ThrRS (the presumed target of borrelidin), a predicted AlaRS and a predicted GlyRS. These are candidates for targeting to multiple compartments and allow us to specifically investigate whether the *Plasmodium* mitochondria harbours tRNA synthetases. No tRNA molecules are encoded by apicomplexan mitochondrial genomes and *Toxoplasma* appears to import tRNA from the cytosol (Esseiva et al., 2004; Pino et al., 2009). How these tRNAs are charged and recycled remains unknown. Here we dissect the trafficking of these three aaRS proteins (*Pf*AlaRS, *Pf*GlyRS and *Pf*ThrRS) using fluorescent protein and epitope tagging approaches, and demonstrate that each is dually targeted to the apicoplast and cytosol but not to the mitochondrion. We show that although the IleRS inhibitor, mupirocin, leads to a specific block in apicoplast division, borrelidin leads to an immediate growth arrest, consistent with inhibition of a *Pf*ThrRS that drives both cytosolic and apicoplast translation.

2. Materials and methods

2.1. Bioinformatic analysis

Sequences of the annotated *Pf*AlaRS (PF13_0354), *Pf*GlyRS (PF14_0198) and *Pf*Thr-RS (PF11_0270) were obtained from PlasmoDB (Aurrecoechea et al., 2009) (www.plasmodb.org). Sequences for putative orthologues were retrieved using BLAST searches (Altschul et al., 1997) of the GenBank non-redundant (nr) (Benson et al., 2011) and orthoMCL databases (Chen et al., 2006). Alignments were performed using ClustalW (Larkin et al., 2007). Analysis of the N-terminal targeting information was performed using SignalP (Bendtsen et al., 2004), PATS (Zuegge et al., 2001) and PlasmoAP (Foth et al., 2003), as well as through manual inspection of residues.

2.2. Parasite culture

Plasmodium falciparum parasites of the 3D7 strain were continuously cultured in O+ human erythrocytes (Australian Red Cross Blood Service, Melbourne, Australia) using a modification of the method established by Trager and Jensen (1976). *Plasmodium falciparum*-infected erythrocytes were maintained in RPMI-HEPES supplemented with 3.6% sodium bicarbonate and 5% Albumax (Invitrogen-Gibco, Australia). The cultures were incubated in an atmosphere of 5% CO_2, 1% O_2 and 94% N_2 at 37 °C. Parasite cultures were synchronised by repeatedly eliminating mature parasites using 5% sorbitol (Lambros and Vanderberg, 1979).

The double-transfectant *P. falciparum* parasites D10 ACP-RFP, CS-YFP were used to simultaneously image the mitochondria and apicoplast in live cells. This double transfectant stably expresses the apicoplast targeting sequence of acyl carrier protein (ACP) fused with red fluorescent protein (RFP) and the mitochondrial targeting sequence of citrate synthetase (CS) fused with yellow fluorescent protein (YFP) (van Dooren et al., 2005). The transfectant cell line was cultured as described above and under drug selection of 5 nM WR99210 and 2.5 μg/ml blasticidin S.

2.3. Drug assays

Parasite growth assays in response to drug treatment were conducted using the SYBR Green assay described by Smilkstein et al. (2004) with modifications as described by Goodman et al. (2007). The multi-drug resistant *P. falciparum* W2mef parasite strain was used for all drug assays. Growth was assayed after 48 h of drug treatment. For delayed death experiments, assays were also performed by treating parasites with inhibitor for 48 h, then replacing with media containing no inhibitor and growing a further 48 h before measuring growth. Compounds were dissolved in methanol, with a final methanol concentration of no greater than 0.1% in culture medium. Experiments were performed in triplicate and were independently performed at least three times.

D10 ACP-RFP, CS-YFP parasites were synchronised, then cultured in approximate 96-h IC_{90} concentrations of mupirocin (200 nM) and borrelidin (5.2 nM), or in 0.1% methanol carrier alone. Parasites were imaged 42 and 90 h after invasion.

2.4. Plasmids and transfection

A triple haemagglutinin (3× HA) tagging plasmid was used to tag the 3′ end of the endogenous copy of PF11_0270, PF13_0354 and PF14_0198 with a 3× HA repeat as previously described (Triglia et al., 2011). C-terminal fragments of PF11_0270 (855 bp), PF13_0354 (1124 bp) and PF14_0198 (849 bp) were amplified from *P. falciparum* 3D7 genomic DNA by PCR using the oligonucleotide pairs [5′gcggccgcGGATGCTCTATACGCAGCAAATG/5′ctgcagcAATGGTTTGGTTTGAGTTAAATTCC], [5′agatctCATCTAAGACGCATGAAGGAAATAATG/ctgcagcTTTGTTAATATTTTTAACATTTCTTCGGCATG] and [5′agatctGTTGGACATGCTGATAGATCAGC/ctgcagcGGAATCCAAAACCTGCTG], respectively. These fragments provide read-through of the native stop codons into the HA tags.

The PF11_0270 PCR fragment was cloned into the 3× HA-tagging plasmid with the *Not*I and *Pst*I sites and the PF13_0354 and PF14_0198 fragments were cloned using the *Bgl*II and *Pst*I sites. Ring stage *P. falciparum* of the 3D7 strain were transfected with 100 μg of purified plasmid DNA. Selection of stable integrants by 3′ single crossover recombination was performed as described by Duraisingh et al. (2002).

The pGlux plasmid was used to tag N-terminal fragments of PF11_0270, PF13_0354 and PF14_0198 with GFP as previously described (Boddey et al., 2009). The N-terminal fragments of PF11_0270 (1–180 bp), PF13_0354 (1–210 bp) and PF14_0198

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