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Research Brief

Babesia bovis: A bipartite signal directs the glutamyl-tRNA synthetase to the apicoplast

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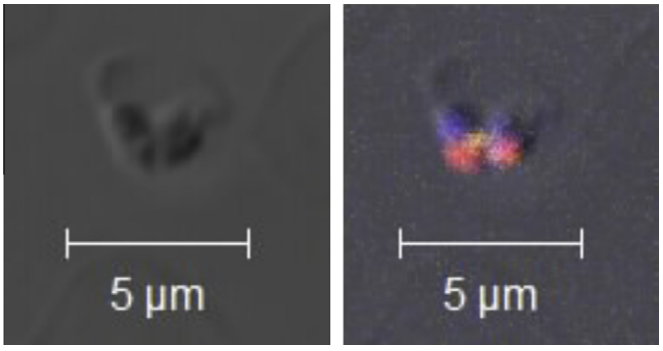
HIGHLIGHTS

- Protein traffic into the apicoplastic lumen requires a bipartite signal.
- Glutamyl tRNA synthetase (GltX) is a putative apicoplast targeted protein.
- GltX contains a bipartite signal sequence, a pre-requisite for apicoplast traffic.
- GltX bipartite sequence successfully directed GFP into the apicoplast.
- Native GltX may utilize its bipartite sequence for apicoplast entry.

GRAPHICAL ABSTRACT



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ABSTRACT

Babesia bovis contains a prokaryotic derived organelle known as the apicoplast. Many participants of the metabolic pathways within the apicoplast are encoded in the nuclear genome and post-translationally imported with the help of a bipartite signal. Recently, an all encompassing algorithm was derived to predict apicoplast targeted proteins for many non-*Plasmodium* apicomplexans in which it reported the presence of 260 apicoplast targeted proteins in *Babesia*. One of these proteins is glutamyl tRNA synthetase (GltX). This study investigates if the putative bipartite signal of GltX alone is sufficient to direct proteins into the apicoplast. Using a transient transfection system consisting of a green fluorescent protein as the reporter, we tested the signal and transit portions of the bipartite signal in apicoplastic transport. We first identified the transcript of *gltX* to be expressed during the asexual blood stages and subsequently confirmed that the complete bipartite signal is responsible for directing the reporter protein into a compartment distinct from the nucleus and the mitochondrion. As GltX bipartite signal successfully guided the reporter protein into the apicoplast, our finding implies that it also directs native GltX into the same organelle.

1. Introduction

The apicoplast is a multi-membranous organelle maintained by some members of the apicomplexan phylum. Due to its prokaryotic origin, it has the potential as a drug target for the control and eradication of diseases such as babesiosis, East Coast fever and malaria (Fichera and Roos, 1997). The organelle's complete function remains unknown. Numerous studies indicate that the apicoplast is indispensable for the parasite's survival (Caballero et al., 2012; Jomaa et al., 1999; Waller et al., 2003; Zuther et al., 1999). Several metabolic pathways have been well documented within the

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apicoplast of the malaria causing *Plasmodium*, although comparative assessments of different apicomplexan genomes suggest that apicoplastic metabolism profile may not be conserved throughout the phylum (Brayton et al., 2007; Gardner et al., 2005; Gardner et al., 2002; Pain et al., 2005). One property that is conserved among apicoplast-containing apicomplexans is that the majority of gene products within this organelle is encoded by the nuclear genome and subsequently subjected to elaborate trafficking mechanism to reach the apicoplast. It was later identified that a bipartite signal is required for most apicoplast targeted proteins (ATPs) (Harb et al., 2004; Tonkin et al., 2008a,b; van Dooren et al., 2002). Software capable of predicting ATPs was developed for *Plasmodium* include PATS (Zuegge et al., 2001) and PlasmoAP (Foth et al., 2003). However, application of either program to predict ATPs in related apicomplexans is unreliable due to the skewed Adenine–Thymidine (AT)-rich genome of *Plasmodium falciparum* used to train PATS and PlasmoAP. Recently, an improved algorithmic program was developed for the remaining apicoplast-containing apicomplexans who are less AT-rich in their genomes. Using the *Babesia bovis* genome data, this software predicted an estimated 260 ATPs in *B. bovis* (Cilingir et al., accepted in PLoS One). Among these ATPs is glutamyl transfer RNA (tRNA) synthetase.

Glutamyl tRNA synthetase (GltX) is a member of the aminoacyl tRNA synthetase family. This is a class of enzymes responsible for the esterification of specific amino acids to their corresponding tRNAs to form aminoacyl tRNAs. These products are used by ribosomes to transfer amino acids onto growing peptides during translation (Woese et al., 2000). The predicted residence of GltX in the *Babesial* apicoplast strongly suggests that active translation occurs within the organelle and further supports work reported in related parasites (Dahl and Rosenthal, 2008; Fleige and Soldati-Favre, 2008).

In this study, we confirm the expression of *gltX* during the asexual blood stages of *B. bovis*. We also identify a predicted GltX bipartite signal sequence *in silico* and determine its function empirically in the traffic of a reporter protein into a cellular compartment distinct from the mitochondrion and nucleus. Our results show the location of the reporter protein to be identical to the native acyl carrier protein, recently shown to also reside within the *Babesia* apicoplast (Caballero et al., 2012).

2. Materials and methods

2.1. B. bovis culture, DNA, RNA and cDNA generation

B. bovis (Mo7 biological clone) was grown in long-term microaerophilic stationary phase culture as previously described (Hines et al., 1989; Levy and Ristic, 1980). Total RNA was isolated using TRIzol (Invitrogen), treated with RNase inhibitor (Roche) and RNase-free DNase (Turbo DNA-free from Ambion) for 30 min at 37 °C. RNA was reverse transcribed with RETROScript kit (Ambion) using oligo-dT primers, according to the manufacturer's instructions for a 2-step RT-PCR.

2.2. Construction of transient transfection plasmids

B. bovis GltX specific primers were designed based on a sequence extracted from accession number BBOV_IV010640. Full length *gltX* transcript was amplified from *B. bovis* cDNA using SuperTaq Polymerase (Ambion) with forward (5′-ATG AAA TTG TAT GCA AAA TTA CTA TAT ACT ATT C-3′) and reverse primers (5′-TTA ATA TTC TAT ATT CGA TAG CTT TGG CTC AG-3′). PCR conditions were 95 °C for 3 min for 1 cycle followed by 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min for a total of 35 cycles and a final elongation step at 72 °C for 10 min. Amplified PCR product was visualized by electrophoresis and subsequently cloned into a pCR®4-TOPO® vector (Invitrogen). Individual clones were selected and sequence confirmed (MacVector vers.11.1).

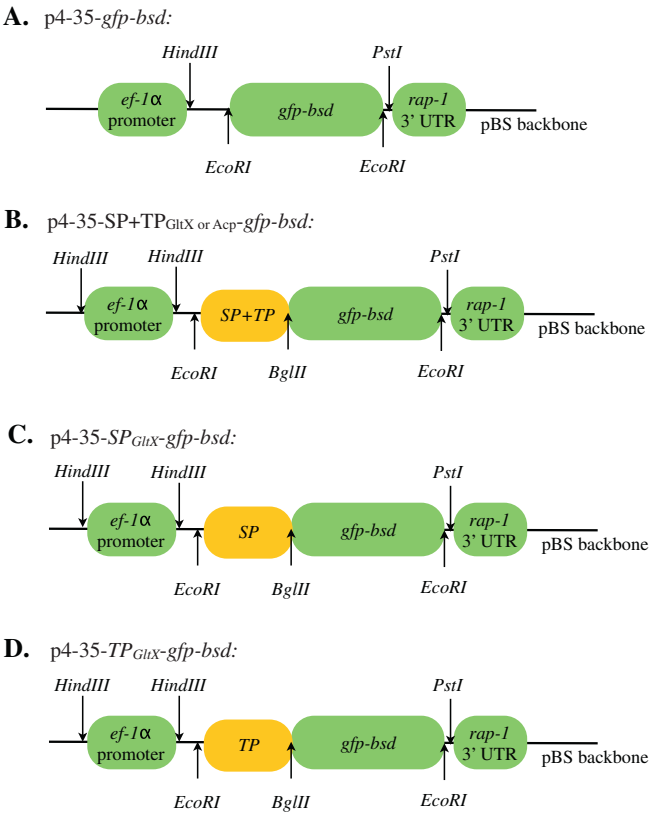

Fig. 1. Schematic diagrams of all plasmid constructs used in the *B. bovis* transfection experiment.

Green fluorescent protein–blasticidin S deaminase (*gfp–bsd*) fusion gene was used as a reporter cassette for the transient transfection system. All the constructs were made similarly as recently described (Caballero et al., 2012). Specifically for this study, the predicted N-terminal bipartite apicoplast target sequence (Signal plus Transit Peptides (SP + TP), 1–23 amino acids) of *B. bovis* GltX was amplified from *B. bovis* cDNA using primers GltX SP (1–23 aa) *EcoRI* forward (5′-GGA ATT C0020ATG AAA TTG TAT GCA AAA TTA C-3′) and GltX TP (24–80 aa) *BglII* reverse (5′-GAA GAT CT TGG TGT GTA TGA TGA AGG ATT ATG-3′). This SPTP fragment was ligated onto the transfection plasmid (Fig. 1A) 5′ to the GFP–BSD cassette, resulting in p4-35-SPTP$_{gltX}$-*gfp–bsd*. Similarly, p4-35-SP$_{gltX}$-*gfp–bsd* was constructed using GltX SP (1–23 aa) *EcoRI* forward (as above) and GltX SP *BglII* reverse (5′-GAA GAT CT TGG TGT GTA TGA TGA AGG ATT ATG-3′) while p4-35-TP$_{gltX}$-*gfp–bsd* was prepared using GltX TP *EcoRI* ATG forward (5′-GGA ATT C ATG ATA AGC TGC TCA AAT AGC TTC-3′) and GltX TP (24–80 aa) *BglII* reverse (as above). All plasmids were purified using the Qiagen endotoxin-free maxiprep kit (Qiagen). Additional plasmids used in the transfection experiment include pBluescript, p4-35-SPTP$_{acp}$-*gfp–bsd* and p4-35-*gfp–bsd* (Fig. 1).

2.3. Transient transfection of B. bovis

Electroporation of *B. bovis*-infected erythrocytes was performed as described by (Suarez and McElwain, 2007) in a Gene Pulser II apparatus (Bio-Rad) using 0.2 cm cuvettes containing 25 µl filter sterilized cytomix buffer (120 µM KCl, 0.15 µM $CaCl_2$, 10 µM K_2HPO_4/KH_2PO_4 pH 7.6, 25 µM HEPES pH 7.6, 2 µM EGTA, 5 µM $MgCl_2$, final pH 7.6) plus 100 µg of the corresponding plasmids