

Title: Investigating mitochondrial protein trafficking in *Crithidia fasciculata*

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Introduction: The twin-arginine translocation (Tat) pathway transports folded proteins across bacterial and plant chloroplast membranes. Bioinformatic analysis uncovered N-terminal, di-arginine motifs in putative mitochondrial proteins of trypanosomatid parasites. We hypothesize that di-arginine motifs are essential for mitochondrial protein import via a Tat system in the trypanosome *Crithidia fasciculata*. A series of *C. fasciculata* proteins, each with known or predicted mitochondrial localization were selected for analysis. Constructs for expression as GFP-fusion protein were designed for the wild-type genes as well as a series of mutants in which codons for di-arginine motifs were replaced with glutamate. Fluorescence microscopy will be used to determine differences of protein location in these mutants relative to GFP-fusion proteins containing wild-type motifs. If a Tat-system mediates protein import to trypanosome mitochondria, this pathway may be an important pathway to pursue for development of anti-trypanosomal drugs for treatment of diseases such as leishmaniasis and Chagas disease.

Method: Wild-type and di-glutamine constructs were developed for six genes: *Isa-2*, *KAP3*, *LYR*, *RISP*, *RNAseH*, and *SODA*. The genes were amplified by PCR from *C.fasciculata* DNA, and di-arginine motifs were replaced with glutamine using modified primers. The genes were ligated into a GFP-fusion vector (pNUS-GFPcH, Tetaud *et al.*, 2002) and transfected into *C. fasciculata*. Following antibiotic selection of transfectants, fluorescence microscopy and Western blot analysis were used to determine subcellular localization and verify expression, respectively.

Results: Transfections of cells carrying the RNAseH di-glutamine (RNAseH-QQ), KAP3-QQ, SODA-QQ, RNAseH wild-type (RNAseH-WT), and GFP constructs were successful. Preliminary analysis of RNAseH-QQ transfectants suggests that the mutant still localizes to mitochondria, but direct comparison to cells expressing the wild-type motif is in progress. The remaining protein targets (*Isa-2*, KAP3-WT, LYR, RISP, SODA-WT) have been successfully cloned and are awaiting ligation into the GFP-fusion vector.

Conclusion: Initial results suggest that di-arginine motifs in RNAseH are not essential for its targeting, but it is unclear if mutant RNAseH successfully traverses the inner membrane or is retained in the intermembrane space. Direct comparison with a wild-type protein will resolve this question. Chemiluminescent analysis of Western blot revealed bands positive for GFP, but this analysis must be repeated to minimize non-specific, antibody-binding. Currently, the remaining cell lines are being validated by immunofluorescence microscopy and Western blot. Identification of a Tat-system in these unicellular eukaryotes may represent a unique aspect of trypanosome cell biology that could be exploited for identifying novel pharmaceuticals to treat illnesses such as Chagas disease and leishmaniasis.