Effects of *Trypanosoma brucei* tryptophanyl-tRNA synthetases silencing by RNA interference

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The kinetoplast genetic code deviates from the universal code in that 90% of mitochondrial tryptophans are specified by UGA instead of UGG codons. A single nucleus-encoded tRNA$^{Trp}$(CCA) is used by both nuclear and mitochondria genes, since all kinetoplast tRNAs are imported into the mitochondria from the cytoplasm. To allow decoding of the mitochondrial UGA codons as tryptophan, the tRNA$^{Trp}$(CCA) anticodon is changed to UCA by an editing event. Two tryptophanyl tRNA synthetases (TrpRSs) have been identified in *Trypanosoma brucei*: TbTrpRS1 and TbTrpRS2 which localize to the cytoplasm and mitochondria respectively. We used inducible RNA interference (RNAi) to assess the role of TbTrpRSs. Our data validates previous observations of TrpRS as potential drug design targets and investigates the RNAi effect on the mitochondria of the parasite.

Key words: tryptophanyl tRNA synthetase - RNA interference - kinetoplastid - *Trypanosoma brucei*

Parasitic protozoa represent a considerable source of human diseases predominant in the tropical and subtropical regions of the globe. Of particular relevance are parasites belonging to the family Trypanosomatidae. *Trypanosoma brucei* and its subspecies are responsible for heavy socioeconomic losses in 36 sub-Saharan Africa countries (1.6 million DALYs) and lead to an estimated 48 thousand deaths per year. Therapy against African sleeping sickness has always been difficult, especially when the disease has reached an advanced stage with central nervous system involvement, as few effective drugs are available. For these reasons, the search and validation of new pharmacological targets against trypanosomiasis is of great concern.

Trypanosomatids are early diverging flagellates that differ from other eukaryotes in a number of features. They contain a remarkable single mitochondrion (the kinetoplast) harboring a large mass of circular DNA molecules concatenated in a unique arrangement, the kinetoplast DNA or kDNA (Lukes et al. 2002). The kinetoplast genetic code, like many other mitochondrial genomes and to the exception of plants, deviates from the universal code in that 90% of tryptophans are specified by UGA instead of UGG codons, although both codons are used in translation. A single nucleus-encoded tRNA$^{Trp}$(CCA) is used by both the nuclear and mitochondrial genes. Since all kinetoplast tRNAs are encoded in the nucleus and later imported into the mitochondria (Lima & Simpson 1996), a C to U deamination event at position 34 of tRNA$^{Trp}$(CCA) changes the anticodon from CCA to UCA allowing the decoding of UGA stop codons to Trp (Alfonzo et al. 1999).

The identification of two tryptophanyl tRNA synthetases (TrpRSs) proteins in *T. brucei* cells was reported (Charrière et al. 2006). TbTrpRS1 (cytoplasmic, accession no. XM821841) and TbTrpRS2 (mitochondrial, accession no. XM820510), where TbTrpRS2 is able of aminoacylate both the unedited tRNA$^{Trp}$(CCA) and the edited tRNA$^{Trp}$(UCA). We used inducible RNA interference (RNAi) in combination with biochemical techniques to assess the role of TbTrpRSs, thereby extending previous observations (Charrière et al. 2006) and validating the TrpRSs as potential drug design targets.

**MATERIALS AND METHODS**

The nucleic acid and amino acid sequences of both genes, TbTrpRS1 and 2, were used to search the *T. brucei* genomic databases, and the results showed no significant identity with other *T. brucei* genes as well as with their human and *Escherichia coli* homologues. A sequence identity of 57% is shared between TbTrpRS1 and 2 distributed along the entire sequence. Due to the low sequence identity, we chose the full length ORF sequences of TbTrpRS1 and 2 for RNAi analysis, based on the idea of increasing the specificity and efficiency of RNAi knockdown (LaCount et al. 2000). *T. brucei* 29-13 procyclic cells were grown in SDM-79 supplemented with 10% fetal bovine serum in the presence of hygromycin (50 µg/ml) and G418 (15 µg/ml) at 25-26°C without agitation (Wang et al. 2000). Each specific DNA for RNAi experiments was amplified by the polymerase chain reaction (PCR) from *T. brucei* 29-13 procyclic genomic DNA using the following gene-specific primers, containing the XhoI and HindIII (underlined) restric-
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For cell morphology analysis, non-induced and tetracycline induction produced a 79\% growth inhibition and complete cell death after 72 h of induction. Both cell lines exhibited eventual complete arrest of growth, which was more quickly achieved in post-induced \textit{TbTrpRS1} cultures (24 h) than in \textit{TbTrpRS2} (72 h), as shown in Figs 1, 2. These cultures showed no recovery after release from tetracycline induction, confirming the lethal and irreversible effect of the gene knockdowns (data not shown).

Expression of \textit{TbTrpRS1} dsRNA led to total degradation of the specific mRNA, which was undetectable by semi-quantitative RT-PCR experiments. RNAi expression against \textit{TbTrpRS1} resulted in severe growth inhibition 4 h after induction (reduction of 84\% relative to control cells) with cell death after 24 h of induction. \textit{TbTrpRS2} knockdown after 6 h of tetracycline induction produced a 79\% growth inhibition and complete cell death after 72 h of induction. Both cell lines exhibited eventual complete arrest of growth, which was more quickly achieved in post-induced \textit{TbTrpRS1} cultures (24 h) than in \textit{TbTrpRS2} (72 h), as shown in Figs 1, 2. These cultures showed no recovery after release from tetracycline induction, confirming the lethal and irreversible effect of the gene knockdowns (data not shown).

Expression of \textit{TbTrpRS1} dsRNA led to total degradation of the specific mRNA, which was undetectable by semi-quantitative RT-PCR. QPCR analysis revealed that transcript levels were reduced more than 16 fold in cells after 24 h of \textit{TbTrpRS1} dsRNA induction (Fig. 2). Expression of \textit{TbTrpRS2} dsRNA also resulted in an efficient degradation of the cognate mRNA resulting in an approximately 3-fold decrease after 48 h of induction and a complete silencing at 72 h of induction, after which \textit{TbTrpRS2} mRNA amounts were not detectable by either semi-quantitative RT-PCR or QPCR (levels < 13 folds) (Fig. 2). These data confirmed the efficiency of silencing of expression of the \textit{TbTrpRSs} by RNAi. Transcript levels were not significantly changed in the non-induced \textit{TbTrpRS2} controls or in the wild type \textit{T. brucei} 29-13 cells ($P = 0.62$). An unrelated transcript for \textit{T. brucei} gGAPDH remained unaffected in the samples analyzed. Furthermore, silencing of one \textit{TbTrpRS} gene did not in-
fluence the expression of the other (data not shown). The non-induced \textit{T. brucei} cells transfected with the \textit{TbTrpRS1} RNAi construct exhibited a small reduction (0.039 folds; \(P = 0.06\)) of the \textit{TbTrpRS1} mRNA as compared to the non-transfected \textit{T. brucei} 29-13 cells. This phenomenon can be explained by a leakage in the T7 dual-promoter system, which has been previously observed (Durand-Dubief et al. 2003).

To analyze the cell morphology, non-induced and tetracycline induced cultures were examined. The phenotype of the control (non-induced) cells appeared normal (Figs 3A, 4A). The distribution of cell morphologies of induced and non-induced knockdowns is presented in the Table. The cells were classified in two categories according to the number of kinetoplasts (K) and nuclei (N) per cell. “Normal” constituted cells with 1K1N or with 2K1N/2K2N, representing different stages of cell division. Cells were classified as “abnormal” when containing a different number of K or N. \textit{TbTrpRS1} knockdown cells also presented a round phenotype and were classified as ‘spheres’. In the control cultures, the population mainly (90-94\%) constituted of 1K1N cells and a minority of dividing cells (2K1N or 2K2N) as shown in Fig. 4A and in the Table.

In the \textit{TbTrpRS1} RNAi cells, morphological abnormalities can be seen after 9 h of tetracycline induction. At 24 h after tetracycline induction the majority of cells (41\%) increased in size, and contained numerous nuclei, kinetoplast, and several flagella, suggesting inhibition of cytokinesis. A significant percentage (33\%) presented the ‘sphere’ phenotype lacking flagella (Table, Fig. 3B). The \textit{TbTrpRS2} knockdown leads to a wide range of morphologic abnormalities. These phenotypes included cells with partially detached flagella (two or more flagella) (Fig. 3B), cells with incomplete cell separations (Fig. 3C), mini-trypanosomes that lack nuclei (Fig. 4D) and cells with defects in cytokinesis (multinucleated; Fig. 4E). At 24 h after tetracycline induction, a high proportion of the cells (36\%) presented abnormalities in their morphology (Figs 3B, 3C, 4B) compared to untreated cells, in which the percentage of abnormal cells was no more than 10\% (Table). The depletion of \textit{TbTrpRS2} resulted in the accumulation of mini-trypanosomes that lack nuclei, suggesting an interruption of mitosis without inhibiting kinetoplast segregation, which led to cytokinesis and cell division and generated enucleated daughter cells (Ploubidou et al. 1999). In Figs 3 and 4 are shown some representative types of cells found in the knockdown of \textit{TbTrpRS1} and 2.
We used the mitochondria-specific stain, Mitotracker (molecular probes), in order to assay functional changes occurring during TbTrpRS2 RNAi induction. A typical reticular pattern is visible throughout the non-induced cells, representing the stained mitochondria, and this pattern is significantly altered after TbTrpRS2 RNAi induction. Induced cells show a decrease in the mitochondria stain, in particular after 48 h of induction (Fig. 4C), indicating dramatic changes of metabolic function. To corroborate these results, the respiration rate or demand of oxygen (DO) was determined using a biological oxygen monitor DM4 (Digimed). The uninduced cells exhibit non-significant changes in their respiration rate compared with the control \((P = 0.789)\). The respiration rate (ppm) was significantly decreased (64\%) at 48 h post-induction (Fig. 5). This result is consistent with the three-fold decrease in TbTrpRS2 mRNA (Fig. 2). After 72 h the DO from these cells was undetectable. The quantitative mRNA analysis from TbTrpRS2 RNAi induced samples suggested that the decrease in the DO and the reduction of the mitochondrial potential occurred after the mRNA depletion (Figs 5, 6). According to these results, we can conclude that slight perturbations in the expression of TbTrpRS2 can trigger significant and immediate alterations in the mitochondrial function and redox potential. In addition, at 72 h post-induction, the complete degradation of specific mRNA in the knockdown cells was compatible with the complete lost of respiration activity and mitochondrial membrane potential (Figs 5, 6). Furthermore, the inhibition of TbTrpRS2 synthesis could decrease the levels of mitochondrial translation, with a decline of mitochondrial redox potential, respiration capability and finally triggering cell death. The TbTrpRS1 RNAi induced deleterious phenotype (Figs 1, 2) prevented an investigation of its role in mitochondrial function, although such a role seems improbable.

**DISCUSSION**

The results we report are consistent with previous observations (Charrière et al. 2006) describing the existence of two TrpRSs genes in *T. brucei* cells and that knockdown of TbTrpRSs by RNAi effectively inhibits the replication of *T. brucei* procyclic forms in culture. In our experiments we extended those observations by the use of full length TbTrpRS1 and 2 genes in the RNAi
constructs. This contributed to a more pronounced sequence-specific knockdown, leading to a clearer physiological effect. Our results show that the outcome of RNA interference on TbTrpRS2 is a rapid decrease of mitochondrial activity, leading to a rapid cell death. The rapid rate at which mutant phenotypes develop after expressing TbTrpRSs dsRNA indicate that these proteins have short half-lives since these enzymes are not abundant in the cells (data not shown). Our results confirm the central role that TbTrpRS1 and 2 play in cell viability, validating these proteins as targets for inhibitor development. In particular TbTrpRS2 is of interest due to its low sequence identity to other TbTrpRSs genes, especially that of humans, and for its role in blocking mitochondrial activity and resulting in cell death.

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