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Involvement of an RNA binding protein containing Alba domain in the stage-specific regulation of beta-amastin expression in *Trypanosoma cruzi*

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ABSTRACT

Amastins are surface glycoproteins, first identified in amastigotes of *T. cruzi* but later found to be expressed in several *Leishmania* species, as well as in *T. cruzi* epimastigotes. Amastins are encoded by a diverse gene family that can be grouped into four subfamilies named α, β, γ, and δ amastins. Differential expression of amastin genes results from regulatory mechanisms involving changes in mRNA stability and/or translational control. Although distinct regulatory elements were identified in the 3′ UTR of *T. cruzi* and *Leishmania* amastin mRNAs, RNA binding proteins involved with amastin gene regulation have only being characterized in *L. infantum* where an Alba-domain protein (LiAlba20) able to bind to the 3′ UTR of a δ-amastin mRNA was identified. Here we investigated the role of TcAlba30, the LiAlba20 homologue in *T. cruzi*, in the post transcriptional regulation of amastin genes. TcAlba30 transcripts are present in all stages of the *T. cruzi* life cycle. RNA immunoprecipitation assays using a transfected cell line expressing a cMyc tagged TcAlba30 revealed that TcAlba30 can interact with β-amastin mRNA. In addition, over-expression of TcAlba30 in epimastigotes resulted in 50% decreased levels of β-amastin mRNAs compared to wild type parasites. Since luciferase assays indicated the presence of regulatory elements in the 3′ UTR of β-amastin mRNA and reduced levels of luciferase mRNA were found in parasites over expressing TcAlba30, we conclude that TcAlba30 acts as a *T. cruzi* RNA binding protein involved in the negative control of β-amastin expression through interactions with its 3′UTR.

1. Introduction

Trypanosoma cruzi is the causative agent of Chagas disease [1], one of the most significant public health challenges in Latin America where almost 8 million people is affected and 28 million people are at risk of contracting the infection [2]. This parasite has a complex life cycle that alternates between a triatomine insect vector and a mammalian host, during which four different developmental stages occur. Replicative non-infective epimastigotes living in the insect midgut migrate through its digestive tract until they reach the hindgut where they differentiate into non-replicative metacyclic trypomastigotes. Infective metacyclic forms released with the insect feces during a blood meal near the bite wound or mucous membrane of the mammalian host are capable of actively invade several types of nucleated cells. Once inside the host cell, trypomastigotes transform into replicative amastigotes that divide in the cytoplasm until they differentiate into bloodstream trypomastigotes. After rupturing the infected mammalian cell, trypomastigotes can reach the bloodstream and invade other cells or can be ingested by the insect vector during a blood meal [3].

T. cruzi adaptation to distinct environments in its vertebrate and invertebrate hosts as well as differentiation into distinct parasite forms requires efficient mechanisms to control gene expression. In trypanosomatids however, gene expression presents many peculiar aspects [4]. Protein coding genes, transcribed by RNA polymerase II do not have canonical promoters and in some cases, they can be also transcribed by RNA polymerase I. Moreover, transcription is polycistronic and, in many cases, different genes that may not be functionally related and are transcribed together in the same polycistron, present highly distinct levels of expression [5].

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Translatable, mature monocystronic mRNAs are generated by coupled reactions of *trans*-splicing and polyadenylation [6–8], which require processing signals present in intergenic regions. Because of this peculiar mode of transcription and processing, the fate of trypanosome mRNAs are mainly controlled at the post-transcriptional level [4,9,10]. Several groups including ours have shown that mRNA half-life and translatability, the major targets of controlling mechanisms, are often dependent on the 3′ untranslated region (3′ UTR) [11–15].

About 50% of the *T. cruzi* genome consists of repetitive sequences, with large gene families encoding surface proteins [16]. One of these multi-gene families encodes amastins, a group of small surface glycoproteins of about 200 amino acids, first identified in amastigotes of *T. cruzi* but later found to be expressed in amastigote forms of several *Leishmania* species [17,18] and in other trypanosomatids, as well as in *T. cruzi* epimastigotes [19,20]. Phylogenetic analyses of different amastin sequences from several trypanosomatid species resulted in a classification consisting of four amastin subfamilies named alfa, beta, gamma and delta-amastins [21]. The initial characterization of amastins as amastigote-specific proteins refers to the *T. cruzi* delta-amastin subfamily, which is organized in clusters associated with tuzin genes not only in *T. cruzi* but also in the genomes of several *Leishmania* species where it has been largely expanded. Similar to other trypanosomatids, the *T. cruzi* genome contains two tandemly arrayed copies of amastin genes belonging to the beta-subfamily, but different from *Leishmania*, where more than 50 delta-amastin genes are present, in the *T. cruzi* CL Brener genome there are only 6 genes belonging to the delta-amastin subfamily [19,21]. Also in contrast to most amastins genes characterized so far, *T. cruzi* beta-amastin transcripts are more abundant in epimastigotes [19]. Aiming at understanding the mechanism underlying amastin developmentally regulation, we and others have identified elements present in the 3′untranslated region (UTR) of delta-amastin genes, which are responsible for their up-regulated expression in *T. cruzi* and *Leishmania* amastigotes using transient [14] and stable transfection assays with luciferase reporter genes [15]. However, there are no reports describing regulatory elements controlling the expression of beta-amastin genes.

Besides the RNA binding protein, named TcUBP1, which has been shown to be involved in interactions with delta-amastin mRNAs [22,23], no other *trans* acting factors that recognize amastin 3′UTR regulatory regions have been identified in *T. cruzi*. In *Leishmania infantum,* where amastin surface proteins represent the largest developmentally regulated gene family reported so far, an Alba domain protein, *Li*Alba20, has been identified as an RNA binding protein that contributes to the amastigote-specific expression of amastin genes [24]. Depletion of this protein leads to a reduced amastin mRNA accumulation in amastigotes [24]. Proteins containing the Alba domain have also been characterized in *Trypanosoma brucei*, where four genes containing such domain were found: ALBA1 (Tb11.02.2040), ALBA2 (Tb11.02.2030), ALBA3 (Tb927.4.2040) and ALBA4 (Tb927.4.2030). Alba3/4 are cytoplasmic RNA-binding proteins that are recruited to stress granules under starvation and co-localize with the RNA interacting protein DHH1 together with poly A+ RNA [25]. Co-immunoprecipitation and pull-down assays revealed the interaction between *T. brucei* Alba proteins with the translational machinery as well as interactions between members of the Alba protein family [26]. *L. infantum* has two Alba-domain proteins, named LiAlba1 and LiAlba3 (also named LiAlba20), which form a complex interacting with other RNA-binding proteins, ribosomal subunits and translation factors [27]. No proteins containing Alba domains have been characterized in *T. cruzi*.

Alba domain proteins (for Acetylation lowers binding affinity), which have shown a great functional plasticity along the evolution, belong to a superfamily identified as the most well characterized architectural DNA-binding protein in Archea [28]. These proteins interact with DNA as dimers [29,30], promote the organization of chromatin at higher levels [31,32] and mediate transcriptional repression [33]. Due to their structure and their ability to bind RNA molecules, it has been postulated that Alba proteins may have multiple functions [34,35]. In *Plasmodium falciparum*, it has been reported that Alba-like proteins can bind to both DNA and RNA with different binding preferences [36,37]. There is also evidence in other eukaryotes, as well as in some members of archeal family, that the role of Alba proteins is related to RNA metabolism [38]. Reversible acetylation of Alba proteins at specific N-terminal lysine residue lowers its binding affinity for double-strand DNA, in a manner reminiscent of the acetylation of the eukaryotic chromosomal proteins [39–41], allowing transcription to proceed [42]. In accordance with studies showing that the activity of Alba proteins can be modulated by phosphorylation, phosphoproteome analysis in *T. cruzi* revealed that Alba proteins (TcAlba30/TcAlba40) can be phosphorylated [43]. Surprisingly, the *Leishmania* Alba-domain proteins also display a flagellar localization and the shuttling of these proteins between the cytoplasm, the nucleolus or the flagellum throughout the parasite development indicates they may fulfil additional roles yet to be characterized [27]. Four genes encoding proteins with Alba domain, annotated as hypothetical proteins are found in the Esmeraldo-like haplotype of the *T. cruzi* CL Brener genome. Here we analysed the role of TcAlba30, the orthologous to LiAlba20 protein, on the stage-specific expression of *T. cruzi* amastins: we found evidence indicating that TcAlba30 is involved in controlling the steady state levels of beta-amastin mRNAs in epimastigotes through elements present in their 3′ UTR.

2. Material and methods

2.1. Phylogenetic analysis

Amino acid sequences of different Alba proteins were aligned with the Muscle algorithm [44] and edited with Jalview editor [45]. A maximum likelihood phylogenetic tree with LG model was built using Mega software [46]. All gap sites were ignored and 1000 bootstrapping replicates were performed. For the tree, indicated trypanosomatid Alba proteins were selected as well as the archeal and eukaryotic representative protein. Resulting phylogenetic tree was visualized using FigTree software available at http://tree.bio.ed.ac.uk/software/figtree/

2.2. Real time PCR

Total RNA was extracted from 2×10^7 exponentially growing epimastigotes, using Trizol (Life Technologies) followed by DNase I treatment with DNA-free (Ambion). cDNA was synthesized from 1 μg of total RNA using Superscript II kit first strand synthesis (Invitrogen) and oligodT primer. In order to quantify retrotranscribed products (qRT-PCR), cDNAs were amplified using TcAlba30 CDS-specific primers (Albafw 5′-AACATTGCGGAGATTGTGAAG-3′ and Albarev 5′-GCCTCGTACTCGTCGTGAATTA-3′); luciferase CDS specific primers (FlucFw 5′-CATAGAACTGCCTGCGTCAGAT-3′ and FlucRev 5′-CGGAATGATTTGATTGCCAAA-3′); β-amastin 390 and β-amastin 394 CDS specific primers (Beta390Fw 5′-TTTGCTGTAGTTGCCGGTATTG-3′and Beta390Rev 5′ACCAAACCCGAGACGGTACTAA-3′ and Beta394fw 5′-TGGACCTTTTGCATACATGGTG-3′ and Beta394rev 5′-TCCATCCAAGTACCCCTTTCCT-3′ respectively). Primer efficiency was evaluated for each pair of primers and all of them were higher than 92%. A control without retrotranscription was also performed as a DNAse I control.