1. Introduction

Differential expression is a result of multiple layers of complex molecular interactions mediated by gene regulatory signals. In particular, the relevance of post-transcriptional regulation of mRNA levels has been largely recognized and the number and nature of the factors, as well as the mechanisms involved, are constantly growing. Kinetoplastids have proved to be interesting models for the study of gene expression regulation, leading to the early discovery of general molecular mechanisms. While post-transcriptional regulation is envisioned as the main step in the control of gene expression, cis elements, trans acting factors, and molecular mechanism involved are not yet understood in depth [1,2].

The protozoan Trypanosoma cruzi is the causative agent of Chagas' disease. This is a major health problem in Latin America, now spreading to areas such as Canada, the US, and Spain, due to migration of infected people into these non-endemic countries [3]. T. cruzi has a complex life cycle alternating through at least 4 stages between 2 hosts: a mammal and an insect. In the mammalian host, the non-replicative metacyclic trypomastigotes, which are highly motile, can invade damaged skin tissue, or mucosa, and can be ingested by macrophages. Inside the cell, metacyclic trypomastigotes differentiate into amastigote forms that divide actively and are released in the mammalian bloodstream as the non-replicative trypomastigote form. Triatominine insects ingest host blood which contains circulating infective bloodstream trypomastigotes. Once in the midgut of the insect, the trypomastigote transforms into the dividing epimastigote stage. At the posterior end of the digestive tract, epimastigotes differentiate to the infective and non-dividing metacyclic trypomastigotes, which are eliminated in the insect feces. To allow for a fast adaptation to the different environments constantly faced by the parasite, a rapid regulation of gene expression is needed [1].

Trypanosomatids display a number of distinctive features regarding mechanisms controlling gene expression. Coding genes are organized in polycistronic transcription units, from where individual mRNAs are processed by trans-splicing and polyadenylation.
genes identified so far [11] and references therein). Primary transcripts from non-related genes adjacent in one polycistronic unit are produced at similar levels. Although, transcription initiation has been associated to polycistronic strand switching and chromatin histones modifications [14] and references therein), regulation of trypanosomatid gene expression is highly dependent on post-transcriptional mechanisms. Thus, quantity, localization and functionality of trans acting factors need to be accurately regulated. RNA-binding proteins (RBPs) are prominent trans acting factors that assist post-transcriptional processes such as mRNA maturations, stability, transport, and translatability [5,6].

Previously, we have identified a T. cruzi RBP (TcRBP19, Acc Number: XP_814431), which is only detected in the amastigote stage showing a predominant cytoplasmatic location [7]. It contains an RNA-binding domain called RNA-recognition motif (RRM). Though some RRM RBPs have been characterized in T. cruzi, their RNA targets as well as their role in gene expression regulation have been reported only for a few cases [8]. TcRBP19 function could not be directly attributed in silico, since orthologues are restricted to kinetoplastids and not characterized so far [7].

As a first approach to understand the differential expression pattern of TcRBP19, we investigated the mechanisms underlying its mRNA abundance. We found that tcrbp19 mRNA is three-fold down-regulated in the epimastigote stage compared to the cellular amastigote stage. In addition, we observed that the tcrbp19 3′ UTR has a down-regulatory effect on the expression of a reporter gene in amastigotes, but not in amastigotes. Using in vitro approaches we demonstrated that the tcrbp19 transcript constitutes a binding target for the TcRBP19 protein itself. We also found that tcrbp19 mRNA stability was decreased when TcRBP19 was ectopically over-expressed in amastigotes. These results suggest the existence of a negative feedback mechanism affecting tcrbp19 mRNA levels that may contribute to the control of TcRBP19 abundance in T. cruzi epimastigotes.

2. Materials and methods

2.1. Parasites

The T. cruzi Dm28 clone [9] was used. Epimastigotes were maintained at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Metacyclic trypomastigotes and in vitro extracellular amastigotes were prepared according to established protocols [10].

2.2. RNA preparation and quantification

Total RNA was extracted from 1 × 10⁸ in vitro differentiated amastigotes or 1 × 10⁹ epimastigotes, using the RNaseasy mini kit (Qiagen) followed by DNase I treatment with DNA-free (Ambion). cDNA was synthesized from 1 μg of total RNA using Reverse Transcription (RT) Kit (Ambion) with an oligo (dT) primers. In order to define the tcrbp19 5′ UTR, spliced leader and tcrbp19 specific primers (SL: 5′-CGCTATTATGTACAGTCTCTG-3′ and TcRBP19rev: 5′-GAGCTACGCGCCGCTATAC-3′) were used.

For the quantification of retrotranscribed products (qRT-PCR), cDNAs were amplified using tcrbp19 CDS-specific primers (TcRBP19Fw: 5′-AGGACATCCAGGTCACAAAC-3′ and TcRBP19rev: 5′-GAGCTACGCGCCGCTATAC-3′) and tcrbp19 5′UTR specific primers (5′UTRfw: 5′-GGCTGAGTTGTGGTGCTCAGGC-3′ and 5′UTRrev: 5′-GGCTGAGTTGTGGTGCTCAGGC-3′) (Fig. S1) in a real time rotary analyzer RotorGene 6000 (Corbett). Relative amounts of the target gene were normalized to the gapdh housekeeping gene using CDS specific primers (GapdhFw: 5′-CGACAC GAGCTGGGGTACT-3′ and GapdhRev: 5′-CTCAACGCTTTGGCACACG-3′). The PCR reaction mixture containing 0.9 μM of each primer, 1× QuantiTect SYBR Green PCR Master Mix (Qiagen), and 2 μL of cDNA template was performed in a final volume of 10 μL. GST-pull down captured mRNAs and total RNA, as well as RNA from wild type and TcRBP19 over-expressing parasites, were compared using the 2 -ΔΔCT method [11].

2.3. Reporter analysis

The pTEX-CAT-3′UTR construct was generated from the pTEX vector [12] with the cat reporter gene (pTEX-CAT), by substitution of the vector reporter 3′UTRs (HindIII and Xhol sites), with tcrbp19 3′UTRs obtained by RT-PCR using specific primers (tcrbp19-1FW_hind: 5′-CGGATTATGTACAGTTCTCAGGAGCC-3′; tcrbp19-1REV_hind: 5′-CTCTGAGCTCCGACCCCAACATTTACACG-3′) (Fig. S1). Both pTEX-CAT-3′UTR and pTEX-CAT were used to transfrect epimastigotes in replica experiments as indicated above and described previously [7]. In order to analyze cat mRNA levels in amastigotes, HeLa cells were infected with transfected parasites as described. Total RNA from the transfected parasites was purified and analyzed by qRT-PCR. Relative amounts of cat mRNA (amplified using primers cat_fw: 5′-CCGGATTATGTACAGTTCTCAGGAGCC-3′ and cat_rev: 5′-GAGCTACGCGCCGCTATAC-3′) normalized to gapdh mRNA, were compared between pTEX-CAT-3′UTR and pTEX-CAT transfected parasites using the 2 -ΔΔCT method [11]. Plasmid copy number was assessed by qPCR measuring the relative amounts of cat DNA over gapdh DNA in each transfectant.

2.4. RNA pull-down assay

Fusion protein GST-TcRBP19 was produced and purified from Escherichia coli cultures as described [7]. Recombinant protein GST-TcRBP19 (or GST alone used as a negative control) was attached to glutathione Sepharose beads (GE Healthcare) for 1 h at 4 °C. 90 μg of total amastigote RNA in binding buffer (10 mM Tris–HCl, 10 mM KCl, 1 mM MgCl₂, 1 mM DTT, 1 mM EDTA) were incubated for 1 h at 4 °C with agitation in the presence of 100 mM heparin and 100 mM spermidine as non-specific competitors. After gently washing with PBS, bound RNA was eluted by a 5 min boiling step. Eluted RNAs were purified using the RNAeasy mini kit (Qiagen) and treated with DNase I (Ambion). TcRBP19 interacting RNA and non-bound RNA were analyzed by qRT-PCR as indicated above. As a negative control we set reactions using tcp566 primers (PufF 5′-CCTGTATTTTCAGGGCATGTCGGAGTGGGACAACATTGCC-3′ and PufR 5′-GTACAGAAAGCTGGGTAAATGCAGATGCAGCATTTATACC-3′).

2.5. Cross-linking and RNA immunoprecipitation (CLIP)

Total protein extract from 1 × 10⁸ over-expressing epimastigotes was prepared as described [13] in the presence of 1,000 U of RNase OUT (Invitrogen) and UV cross-linked for 30 min at 254 nm. Cross-linked RNA-protein complexes were immunoprecipitated with anti-TcRBP19 polyclonal antibody overnight and then, 150 μL Protein A Sepharose (GE Healthcare) was added. A control reaction without antibody addition was also performed. Ribonucleoprotein complexes were washed three times with 200 μL of binding buffer (10 mM Tris–HCl, 10 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA). DNase treatment was performed using DNA free kit (Ambion).

cDNA was prepared from immunoprecipitated RNA and RT-PCR was done using Tc19lp1Fw: 5′-TGAGGATTATCGCAGCGCCCATAC-3′ and oligoD primers.
2.6. TcRBP19 ectopic over-expression

The plasmid pTEX-TcRBP19 was constructed using the pTEX plasmid [12], in which the tcrbp19 open reading frame was inserted using the BamHI and XhoI sites. Two biological replicas of electroporated epimastigotes with each plasmid (pTEX-TcRBP19 and pTEX alone) were cultured in the presence of Genetecin 500 μg/ml, and checked by optical microscopy and Western blot using the specific antibody anti-TcRBP19, as previously described [7].

2.7. TcRBP19 target mRNA stability assays

Actinomycin D (Sigma) was added to TcRBP19 over-expressing and control pTEX transfected epimastigote cultures (2 × 10⁷ cells/mL) at a 10 μg/mL concentration. Two milliliter aliquots were removed at different time points (0, 10, 20, 40 and 80 min), and RNA was extracted as described above. The primers for qRT-PCR were designed to anneal with the tcrbp19 5’UTR region to ensure the exclusive amplification of the endogenous tcrbp19 mRNA. The fold change of the endogenous tcrbp19 normalized to gapdh and relative to the expression at time zero was calculated using the 2⁻ΔΔCT method as described previously [11].

3. Results and discussion

3.1. The tcrbp19 mRNA is down-regulated in T. cruzi epimastigotes

Trying to get an insight into the molecular mechanisms involved in the differential expression of the TcRBP19 protein in T. cruzi, we studied the tcrbp19 transcript abundance. Despite failure to detect TcRBP19 protein, we found tcrbp19 transcripts in the epimastigote stage. This is not surprising due to the constitutive transcription of protein genes in kinetoplastids. Sequencing analysis of the amplified products using primers directed to the gene and the spliced leader or polyA tail enabled us to experimentally define the 5’ and 3’ UTR regions (Fig. S1).

Mirroring the protein profile, quantification of the tcrbp19 steady-state mRNA level revealed a down-regulation in epimastigotes compared to amastigotes (Fig. 1). In accordance with this observation, transcriptome analysis has revealed a diminished content of tcrbp19 mRNA in epimastigotes compared to the cumulative expression in the four developmental parasite life cycle stages [14]. Even though the involvement of translational and post-translational processes may also contribute to the actual protein content, this finding points out to the existence of a mRNA stability mechanism acting on the regulation of the differential expression and provide a possible explanation for the TcRBP19 protein pattern previously observed [7].

3.2. The tcrbp19 3’UTR down-regulates reporter gene transcripts in T. cruzi epimastigotes

It is widely recognized that the cis signals that participate in the control of mRNA stability are mainly located at the 3’UTRs. Such is also the case for T. cruzi, where the regulatory role of the 3’UTRs of some protein coding genes, differentially expressed during the parasite life cycle has been already reported [2,15–18]. Therefore, we studied the putative involvement of the tcrbp19 3’UTR in its post-transcriptional regulation. To address this issue, we analyzed the expression of a reporter gene (cat) from an episomal vector (pTEX) both in the epimastigote and the amastigote stage. Reporter mRNA levels were compared between two transfected populations: pTEX-CAT, which bears the gapdh 3’UTR and pTEX-CAT-3’UTR, carrying the tcrbp19 3’UTR downstream the reporter coding region. Epimastigote transfected parasites were cultured at low antibiotic pressure to maintain a low copy number of plasmid and, hence, minimize the effect of over-expression. Nonetheless, plasmid copy number per parasite was calculated from total DNA by qPCR using cat and gapdh specific primers, yielding in average, one copy of the vector per transfected genome.

The effect of tcrbp19 3’UTR on the abundance of cat mRNA, was quantified by qRT-PCR using gapdh as a control (Fig. 2). We found that in epimastigotes, the substitution of the reporter gene 3’UTR by the tcrbp19 3’UTR significantly reduces reporter mRNA levels. On the contrary, in the amastigote stage, a significant increase of cat mRNA levels comparing data from pTEX-CAT-3’UTR with the pTEX-CAT parasites is found. These results supports the existence of a regulatory element in the tcrbp19 3’UTR that remains to be delimited.

Though the tcrbp19 3’UTR contains U-rich tracts, a sole AU rich core element AUUUAA (ARE) was found. This 3’UTR element, widespread in eukaryotes, has been involved in the regulation of mRNA stability [19,20]. In T. cruzi, the role of AREs in the 3’UTR of TcSMUG mRNA mediating developmental regulation has been suggested [21,22]. Besides, a 3’UTR 43-nt U rich element, which also includes an ARE core pentamer, has been involved in the modulation of mRNA abundance in the intracellular amastigote stage [23]. However, no significant similarity of the tcrbp19 3’UTR to this 43-nt U element was found. This is not surprising since different subset of genes responding to different signals may have similar patterns of expression during development.

3.3. The tcrbp19 transcript constitutes a TcRBP19 target

Previous analysis of the in vitro amastigote RNA targets of TcRBP19 protein by GST pull-down assays followed by competitive hybridization of the eluted and bound RNAs to a T. cruzi DNA microarray (to be published elsewhere), revealed tcrbp19 as the most represented mRNA. Since these data suggest that the TcRBP19 protein may assist its own mRNA metabolism, we here focused on this interaction.

Firstly, the amounts of tcrbp19 relative to gapdh obtained from amastigote total or captured RNA in the TcRBP19 GST pull-down were quantified (Fig. 3A) to verify those previous results. As a control, the relative amount of the unrelated tcpuf6 transcript [24] was also determined. The specific detection of tcrbp19 mRNA in the pull-down supports that TcRBP19 protein associates with its own endogenous mRNA in vitro.

Secondly, to confirm the direct interaction of TcRBP19 with its encoding mRNA, we performed CLIP assays with total extract from TcRBP19 over-expressing parasites. The ectopic over-expression
was achieved by cloning the TcRBP19 coding region in the pTEX vector (pTEX-TcRBP19) (Fig. S2). Cross-linking followed by immunoprecipitation, using a previously characterized specific TcRBP19 antibody [7], enabled the detection of tcrbp19 mRNA in the immunoprecipitated fraction (Fig. 3B).

3.4. The endogenous tcrbp19 mRNA is down-regulated when TcRBP19 expression is forced in epimastigotes

Since no functional RNA interference mechanism has been demonstrated in T. cruzi [25] and only a few cases of gene knock down have been reported in this parasite, over-expression constitutes a major approach to study protein function. Indeed, ectopic over-expression is helping to understand RBP functions/mechanisms in trypanosomatids (for a recent example see [26]). In order to understand the biological significance of TcRBP19 binding to its own mRNA, we studied the effect of its over-production. In the TcRBP19 over-expressing parasites the level and functionality of the ectopic tcrbp19 transcript relies under gapdh UTR and intergenic region control (Fig. S2). As previously reported, the forced over-expression of TcRBP19 did not produce any evident morphological changes in T. cruzi epimastigotes [7]. However, under these conditions, a significant fall in the endogenous steady-state of tcrbp19 transcript relative to gapdh mRNA levels was observed (≈150-fold, \( p < 0.05 \)). For the unequivocal quantification of the endogenous tcrbp19 transcripts, primers driving the amplification of part of the tcrbp19 5’UTR, absent in the TcRBP19 over-expressing vector, were designed. In addition, the amount of endogenous tcrbp19 transcript relative to gapdh gene transcript was measured by qRT-PCR at different time points after actinomycin D treatment, and normalized to time 0 (Fig. 4). A decrease of the endogenous tcrbp19 mRNA stability can be deduced for TcRBP19 over-expressing parasites when compared to the pTEX control ones. Therefore, the involvement of TcRBP19 in the destabilization of its cognate transcript is strongly suggested.

Fig. 2. Effect of tcrbp19 3’UTR on reporter gene mRNA steady state levels. mRNA quantification relative to gapdh mRNA was assessed by qRT-PCR in total RNA obtained from parasites transfected with pTEX-CAT-3’UTR and control pTEX-CAT, normalized to the latter both in T. cruzi epimastigotes (A) and amastigotes (B). Each point corresponds to the average obtained from triplicates of parasite populations derived from two independent pTEX-CAT-3’UTR plasmid transfection and sd. values are indicated. Normality and homogeneity of the data were evaluated by analysis of variance (ANOVA). t-test was used to determine the statistical significance. “Statistical significance difference (\( \frac{\alpha}{\beta} p < 0.005 \).”

Fig. 3. Binding of tcrbp19 mRNA to TcRBP19 protein. (A) Quantification of tcrbp19 mRNA bound in vitro to the recombinant GST-TcRBP19. qRT-PCR analysis of tcrbp19 transcript relative to gapdh mRNA was assayed in total cDNA and in the pull-down (pd) fraction. The amount of the unrelated tcpuf6 transcript was determined as a negative control. Average values of triplicates are shown and the corresponding sd. are indicated. Normality and homogeneity of the data were evaluated by analysis of variance (ANOVA). t-test was used to determine the statistical significance. “Statistical significance difference (\( \frac{\alpha}{\beta} p < 0.005; \frac{\alpha}{\beta} p < 0.0001 \).) (B) Protein–RNA crosslinking and immunoprecipitation with (α-TcRBP19) or without (-) anti-TcRBP19 affinity purified polyclonal antibody was performed on TcRBP19 over-expressing epimastigotes, followed by RT-PCR using the primers shown in the upper scheme. MWM: Molecular Weight Marker GeneRuler™ 100 bp DNA Ladder (Fermentas™).

Fig. 4. Effect of TcRBP19 over-expression on tcrbp19 mRNA stability. The amount of endogenous tcrbp19 transcript was determined in triplicates at different time points after actinomycin D treatment by qRT-PCR in TcRBP19 over-expressing epimastigotes (pTEX-TcRBP19), using tcrbp19 5’UTR primers (filled circles). Control parasites transfected with pTEX only were processed in the same way (empty circles). mRNA quantification was calculated relative to gapdh mRNA and normalized to time 0 as described [11].
Negative feedback of mRNA by their encoded products provides a fine-tuning mechanism for the precise control of their expression. In higher eukaryotes, some RBPs coding genes have been involved in these regulatory mechanisms modulating transcription, alternative splicing, 3’ end processing and translation efficiency (see for example [27–31]) establishing auto- or hierarchical regulation. Since trypanosomes do not regulate transcription initiation [5,6], control of mRNA abundance is entirely dependent on post-transcriptional events among which, exploiting mRNA down-auto-regulation constitutes a plausible recourse.

We have very recently shown that over-expression of TcRBP19 in T. cruzi impairs differentiation into the metacyclic infective form and subsequent host cell infectivity [32]. Thus, to avoid impairment of progression through the parasite’s life cycle, TcRBP19 levels in the epimastigote stage must remain tightly controlled. In this context, the specific down-autoregulatory mechanism here revealed could serve to monitor the tcrbp19 mRNA levels in the epimastigote stage. This mechanism might enable a close tracking of TcRBP19 dose granting its under representation in epimastigotes.

The delimitation of the 3’UTR elements required for mRNA modulation, as well as the study of stage-specific TcRBP19 mRNA targets and protein partners would contribute to further understand TcRBP19 function.

In conclusion, our work not only shows that the abundance of tcrbp19 mRNA in T. cruzi epimastigotes is controlled via its 3’UTR, but also identifies its protein product, TcRBP19, as a trans acting factor exerting a negative control on tcrbp19 mRNA stability in T. cruzi epimastigotes. This finding underscores the importance of RBPs and their participation in multiple layers of control of gene expression, being TcRBP19 the first reported protein that is involved in mRNA negative autoregulation in trypanosomatids.

Acknowledgments

The authors are grateful to Dr. María Ana Duhagon and Dr. Martín Ciganda for critically reading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.096.

References