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Biochemical and Biophysical Research Communications 319 (2004) 169–177
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TcZFP1: a CCCH zinc finger protein of *Trypanosoma cruzi* that binds poly-C oligoribonucleotides in vitro

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Received 16 February 2004
Available online 12 May 2004

Abstract

We have identified two zinc finger proteins of *Trypanosoma cruzi*, the protozoan parasite that causes Chagas disease in humans. These proteins, named tcZFP1 and tcZFP2, share the unusual zinc finger motif (CCCH) found in a diverse range of RNA-binding proteins involved in various aspects of the control of cell homeostasis and differentiation. We report here the functional expression of a recombinant tcZFP1, and the relative affinity and stability of the specific complexes formed between the protein and synthetic oligoribonucleotides containing C-rich sequences.

Keywords: *Trypanosoma cruzi*; CCCH zinc finger protein; Metacyclogenesis; EMSA

The protozoan parasite *Trypanosoma cruzi* is the causative agent of American trypanosomiasis, or Chagas disease, which affects millions of people in Central and South America. Its life cycle involves at least three distinct developmental stages: epimastigotes, trypomastigotes, and amastigotes. The epimastigote forms replicate within the insect host midgut and change into the non-replicative metacyclic trypomastigote forms by a process named metacyclogenesis. Metacyclic trypomastigotes are released in the excreta of the reduviidae insects (triatomine) during their blood meal and can invade the mammalian host cells. Once inside host cells, the parasite differentiates into the replicative amastigote form. Finally, this form differentiates into bloodstream trypomastigotes, which infect new cells [1,2].

Trypanosoma cruzi, like other trypanosomatids, uses gene expression control mechanisms that are not encountered in most eukaryotes. Unlike in higher eukaryotes, gene expression in this parasite is mainly regulated by post-transcriptional processes that involve polycistronic mRNA maturation or mRNA stability and translation in a stage-specific manner [3]. The gene regulation mechanisms acting on mRNA stability are probably critical for *T. cruzi*, as the parasite is able to adapt rapidly to changes in environmental conditions (e.g., temperature, nutrient availability, and host defense mechanisms) encountered during its life cycle. In eukaryotes, mRNA decay rates are determined by the interaction between *cis*-acting sequences or structural elements within the mRNA and specific RNA-binding proteins [4,5]. Most *cis*-acting elements, such as AU-rich elements (ARE) and polypyrimidine tracts, are located within the 3′-untranslated region (UTR) of eukaryotic mRNAs. Many of these elements are involved in mRNA destabilization [6–8].

The stability of many developmentally regulated transcripts in differentiating trypanosomatids appears to be determined, at least in part, by structural elements

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doi:10.1016/j.bbrc.2004.04.162

and specific *cis*-acting sequences within the 3′-UTR of the mRNAs, which function as either regulatory or cleavage targets [9–16]. Some of these sequences present features of AREs [3,17,18] and might target the mRNA to the exosome for degradation [19]. Interestingly, the expression of the gene encoding human HuR in *Trypanosoma brucei* increases the abundance of a set of unstable mRNAs containing ARE-like elements in the bloodstream form of the parasite [20].

Furthermore, some *cis*-acting elements, like a conserved 450-nucleotide element present in the 3′-UTR of a number of *Leishmania* mRNAs, have been shown to mediate the production of some amastigote-specific mRNAs by a translational control mechanism rather than by controlling mRNA stability [21].

In recent years, research has focused on the identification of the *trans*-acting factors that interact with sequence motifs in mRNAs and modulate translational efficiency or mRNA stability in kinetoplastids. In *T. cruzi*, members of an RNA recognition motif (RRM)-type RNA-binding protein family, named TcRBP, have been shown to interact with specific transcripts. Some of the genes encoding members of this family show a developmentally regulated expression pattern that might be related to the stage-specific turnover of specific transcripts [22]. Two of these proteins, TcUBP1 and TcUBP2, were previously shown to be U-rich RNA-binding proteins [23]. TcUBP1 also recognizes the 44-nucleotide AU-rich element within the 3′-UTR of the *T. cruzi* small mucin (TcSMUG) RNA. This element destabilizes mucin mRNA in a stage-specific fashion [24]. Recently, two proteins (tbZFP1 and tbZFP2) containing a CCCH zinc finger motif $[C(X)_8C(X)_5C(X)_3H]$, a characteristic feature of some RNA-binding proteins, were identified in *T. brucei*. These proteins are involved in the control of *T. brucei* differentiation to the procyclic form [25]. Members of this family have also been found in other eukaryotic species [26–29]. They are heterogeneous regarding the number of CCCH motifs contained and exert their roles on the mRNA molecule in different manners. Recently, tristetraprolin, a member of a small family bearing two copies of the unusual CCCH zinc finger domain, was found to destabilize ARE-containing mRNAs, such as cytokine and growth factor mRNAs [28–31], in a CCCH domain-dependent fashion [28].

Here, we report the characterization of the *T. cruzi* homolog of tbZFP1, named tcZFP1. Unexpectedly, this protein recognizes C-rich ribopolymers in vitro instead of ARE motifs. The *tcZFP1* gene is differentially expressed throughout metacyclogenesis. Hence, it might be involved in modulating stage-specific mRNA decay pathways and mRNA accessibility to the translational machinery, mechanisms that are ultimately involved in the differentiation of infective parasite stages.

Materials and methods

Cloning, sequencing, and analysis of the TcZFP1 and TcZFP2 genes

A BLAST search was performed using the sequences of *tbZFP1* (GenBank Accession No. AY049059) and tbZFP2 (GenBank Accession No. AAK39107) from *T. brucei* to identify *T. cruzi* homologs deposited in the GenBank database. ESTs from *T. cruzi* found to share homology with *tbZFP1* were used to create a contig containing the entire coding sequence of the *tcZFP1* and *tcZFP2* genes. The coding region of *tcZFP1* was amplified by PCR from the genome of *T. cruzi* clone Dm28c [32], using the set of primers ZFP1f (5′-GGGGG GATCCATGCAGCCCCAAACACCCACA-3′) and ZFP1r (5′-GGG AAGCTTGATTCGAGGGATATGTTTTTTCAG-3′), whereas the coding region of *tcZFP2* was amplified using primers ZFP2f (5′-AT GCAGGGGTATTTTGCACTCAACCAACC-3′) and ZFP2r (5′-TT ATGACGCCGGCGTTTCTCCCT-3′). The PCRs were carried out in a 100 μl reaction volume, containing 20 pmol of the primers ZFP1f and ZFP1r or ZFP2f and ZFP2r, 200 μM each dNTP, 1.5 mM $MgCl_2$, 1× *Taq* DNA polymerase buffer, 2.5 U *Taq* DNA polymerase (Invitrogen), and 100 ng *T. cruzi* total DNA, purified as described in [33]. PCR was performed on a Perkin–Elmer 9700 thermal cycler with a hot start at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. The PCR product was cloned directly into pCR-TOPO (Invitrogen), according to the manufacturer's instructions. Recombinant plasmids were prepared with a plasmid purification kit (Qiagen) and sequenced on an Applied Biosystems automated DNA sequencer (model 3100). The DNA sequences were analyzed with the EditSeq and MegAlign programs from the Lasergene sequence analysis package (DNASTAR).

Expression and purification of a recombinant tcZFP1 protein

Construction of the pGFPXa vector. An expression vector, using pQE30 (Qiagen) as a backbone, was constructed such that tcZFP1 was fused with the green fluorescent protein (GFP). pEGFP-C3 (Clontech) was used as a template to amplify the coding region of the GFP gene in a 100 μl PCR in the following conditions: 10 pmol of foward and reverse primer for the GFP gene (GFPf, 5′-GGGGGATCCATG GTGAGCAAGGGCGAGGAGC-3′ and GFPr, 5′-CGCGGAAGC TT**GAATTC**ACCCCGACCTTCGATGA GCTCGAGATCTGA-3′), 200 μM each dNTP, 10× *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Invitrogen). *Bam*HI and *Hin*dIII sites were added to the 5′ end of primers GFPf and GFPr, respectively. In addition, the GFPr primer contained an *Eco*RI site (bold) and the sequence encoding the factor Xa recognition site (underlined). The PCR consisted of a denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The PCR product was cloned into the *Bam*HI and *Hin*dIII sites of pQE 30 in which the unique *Eco*RI site had been removed previously by digestion with *Eco*RI followed by a fill-in reaction with the Klenow enzyme. This new plasmid was named pGFPXa.

Cloning of the TcZFP1 gene in pGFPXa. Primers tcZFPf (5′-AG GAAGAATTCATGCAGCCCCAAACACA-3′) and tcZFPr (5′-TC GCCGAATTCTCAGCCCCCGTT-3′) were used to amplify the coding region of *tcZFP1*, under the following conditions: 100 ng of total DNA from *T. cruzi* Dm28c, 10 pmol of each primer tcZFPf and tcZFPr, 200 M each dNTP, 1.5 mM $MgCl_2$, 10× Taq DNA polymerase buffer, and 2.5 U *Taq* DNA polymerase (Invitrogen). The reaction mixture was heated for 4 min at 94 °C, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The PCR-amplified DNA fragment was purified using the high pure PCR product purification kit (Roche), digested with *Eco*RI, and cloned into the *Eco*RI site at the 3′ end of the GFP gene within pGFPXa. *Escherichia coli* M15 was transformed with the

resulting product. Clones containing the *tcZFP1* gene in the correct orientation were selected by colony PCR, using the primers GFPf and tcZFPr.

Expression of the GFP–tcZFP1 fusion protein

The His_6-tagged GFP–tcZFP fusion protein was expressed in *E. coli* M15 by adding IPTG (final concentration = 2 mM) and allowing the culture to grow for an additional 3–4 h at 28 °C. The recombinant protein was purified in native conditions using the Ni–NTA spin kit (Qiagen), according to the manufacturer's recommended protocol. Samples of total extracts of uninduced and IPTG-induced *E. coli* cultures as well as the purified GFP-tcZFP1 fusion protein were fractioned on SDS–PAGE and blotted onto nitrocellulose as described [34]. Filter was blocked with TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% non-fat milk for 30 min at room temperature and incubated with anti-His tag monoclonal antibody (Amersham Biosciences) according to manufacturer's instructions. After washing with TBST/milk, the filter was incubated with goat anti-mouse IgG, conjugated to alkaline phosphatase (Bio-Rad), and washed and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega) as recommended by manufacturer.

Trypanosome cultures

Epimastigotes of *T. cruzi* Dm28c were cultured in LIT medium at 28 °C [32]. *T. cruzi* epimastigotes were allowed to differentiate in chemically defined conditions (TAU3AAG medium), as previously described [32]. Briefly, epimastigotes in late exponential growth phase were harvested by centrifugation and incubated for 2 h in triatomine artificial urine (TAU, 190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 8 mM sodium phosphate buffer, pH 6.0) at a density of 5×10^8 cells ml^{-1}. They were then used to inoculate cell culture flasks containing TAU3AAG (TAU supplemented with 50 mM sodium glutamate, 10 mM L-proline, 2 mM sodium aspartate, and 10 mM glucose), at a density of 5×10^6 cells ml^{-1} at 28 °C. Differentiating epimastigotes (cells adherent after 24 h) were obtained by discarding the supernatant and vigorously shaking with TAU3AAG at room temperature to detach the adherent epimastigotes from the culture flask.

Polysomes, RNA isolation

Polysomal *T. cruzi* RNA was obtained as described previously [35]. The RNA was treated with RNase-free DNase and used for Northern blot analysis. Total RNA was extracted from *T. cruzi* by a scaled-up version of the simultaneous DNA and RNA isolation procedure described in [36].

Northern blot analysis

Northern blot analysis was performed according to standard protocol [34]. A 339-bp DNA fragment corresponding to the *tcZFP1* coding region was used as a probe (1×10^9 cpm μg^{-1}). This fragment was radioactively labeled with [α-^{32}P]dCTP, using the random primer DNA labeling system (Invitrogen) and purified through a probeQuant G-50 microcolumn (Amersham Biosciences). Hybridization was performed in hybridization solution (40% formamide, 2× Denhardt's solution, 5× SCC, 01% SDS, and 100 μg/ml salmon sperm DNA) overnight at 42 °C. Following hybridization, the membrane was washed successively with 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.5× SSC/0.1% SDS at 42 °C and used to expose X-ray Hyperfilm MP (Amersham Biosciences).

Oligoribonucleotide design and labeling

Oligoribonucleotides were from Qiagen Operon. The four homoribopolymers were 15 nt long. The other oligoribonucleotides used were as follows: ARE (5′-AUUUAUUUAUUUAUUUA-3′), CU (5′-CUUUCUUUCUUUCUUUC-3′), GU (5′-GUUUGUUUGUUUGUUUG-3′), and U-rich 26mer (5′-UAAUAUUUUUUCGUUAUAUUUUUUUG-3′).

The probes were all end-labeled with T4 polynucleotide kinase (Roche) and [γ-^{32}P]ATP (Amersham Biosciences) as described [34].

Binding reactions and electrophoretic mobility assays

The specified protein concentration and 10–20,000 cpm of the probe were incubated at room temperature for 20 min in a 20-μl reaction volume in binding buffer (10 mM Tris–HCl, pH 8.0, 10 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT) supplemented with 5 mM spermidine and 1 μg heparin as a non-specific competitor and immediately loaded onto a 6% native polyacrylamide gel. Specific competitors were added and the mixture was incubated at room temperature for 10 min before adding the labeled probe. For the run-off experiments, after complex formation, a 1000-fold molar excess of unlabeled oligoribonucleotide was added to prevent the dissociating proteins from rebinding to the labeled probe. At different time points, aliquots were removed and run out on a gel to determine the amount of complex remaining. Quantitative measurements were obtained by scintillation counting in a β-counter. Each lane in the gel was cut into several 1-cm long pieces and liquid scintillator was added. Plots of radioactivity retained by the complex relative to time 0 were analyzed to derive kinetic constants.

Results

Cloning and characterization of TcZFP1 gene

Two genes encoding novel proteins with CCCH motifs (*tbZFP1* and *tbZFP2*) have been characterized in *T. brucei* [25]. The presence of the CCCH motif suggested that these proteins interact with RNAs, as is the case for other proteins bearing this motif [29]. As CCCH proteins might also be important during *T. cruzi* differentiation, we decided to search for homologous genes in *T. cruzi*. A search of the sequence database using the *tbZFP1* gene as the query sequence identified four ESTs (GenBank Accession Nos. AA908048, AI562549, AI073312, and AA958110). Likewise, a BLAST search using the *tbZFP2* sequence revealed four *T. cruzi* ESTs (GenBank Accession Nos. AI622988, AI066121, AI065287, and AI053368). The ESTs from *T. cruzi* and *tbZFP1* and *tbZFP2* were aligned to determine the coding region of the *tcZFP1* and *tcZFP2* genes, respectively. Primers were constructed to amplify these genes from the *T. cruzi* Dm28c genome. *TcZFP1* (GenBank Accession No. AY333787) encodes a small protein of 112 amino acid residues that shares approximately 64% similarity with tbZFP1 (Fig. 1). *TcZFP2* (GenBank Accession No. AY333788) encodes a protein comprising 138 amino acids, sharing 34% similarity with tbZFP2. However, tcZFP2 and tbZFP2 share structural conservation of the CCCH motif and the WW domain of protein–protein interaction (Fig. 1). The most striking difference between them is a glycine-rich domain

upstream of the CCCH motif in tcZFP2 that is not present in the *T. brucei* homolog (Fig. 1).

Analysis of the expression of TcZFP1

Previous work indicated that the expression of several *T.cruzi* genes is regulated through the differential mobilization of their respective mRNAs to the polysomes [35,37,38]. We first analyzed the role of *tcZFP1* as a previous microarray study of gene expression during *T. cruzi* metacyclogenesis (Krieger et al., in preparation) which showed that the expression of *tcZFP1* is developmentally regulated. The differential expression of *tcZFP1* was confirmed by Northern blot analysis of total and polysomal RNA fractions. Northern blot showed that the level of *tcZFP1* mRNA in the polysomal fraction increased when the parasite was submitted to nutritional stress for 2 h in TAU medium, which triggers *T. cruzi* metacyclogenesis (Fig. 2). This indicates that more tcZFP1 might be required at this stage.

TcZFP1 selectively binds a poly-C homopolymer

Proteins containing the zinc finger motif with the CCCH structure have been found to bind to RNA. To assess the ability of tcZFP1 to bind RNA, we subjected the recombinant protein and different RNA probes to the electrophoretic mobility shift assay (EMSA). TcZFP1 was fused with GFP (43 kDa) in order to improve its solubility in *E. coli*. It has been reported that GFP tagging allows the monitoring of protein expression and increases protein stability and solubility in bacteria [39]. Expression was induced with 2 mM IPTG

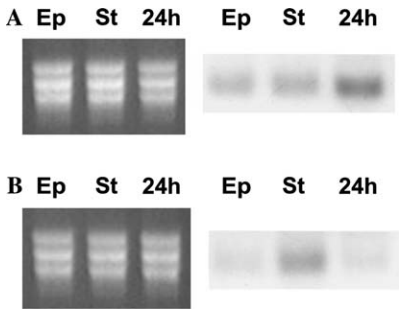

Fig. 2. Northern blot analysis of *TcZFP1* RNA levels in *T. cruzi*. Total (A) and polysomal (B) RNAs were extracted from epimastigotes (Ep), epimastigotes under nutritional stress (St), and differentiating epimastigotes (24 h) and subjected to electrophoresis in a denaturing agarose gel, blotted onto nitrocellulose, and hybridized with the radioactively labeled probe.

and the recombinant protein purified on nickel–NTA resin (Fig. 3A). The induced and non-induced *E. coli* extracts as well as the purified recombinant protein were reacted against the anti-His tag monoclonal to confirm the identity of the purified protein (Fig. 3B). Bacterial cultures expressing the GFP–tcZFP1 fusion protein became green after addition of IPTG, indicating that the overexpressed GFP carrier was functional and so might be the tcZFP1.

We tested the binding specificity of GFP–tcZFP1 by using different oligoribonucleotides as probes. First, EMSA was performed with the four labeled homopolymers. In our experimental conditions, binding was observed only with the poly-C sequence (Fig. 4A). As expected, the same unlabeled probe completely eliminated the labeled complex in competition experiments.

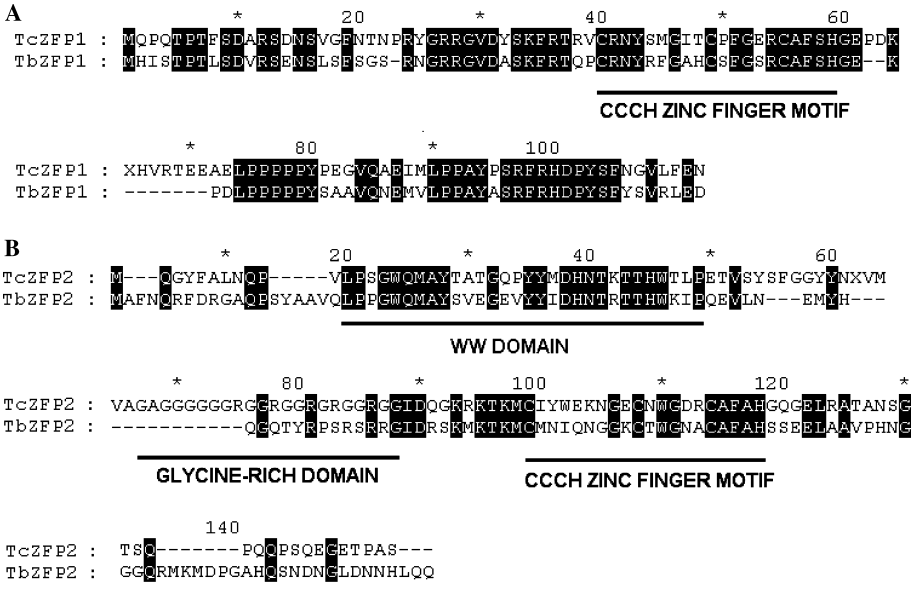

Fig. 1. Comparison of the amino acid sequences of the TcZFP1 (A) and TcZFP2 (B) proteins and their *T. brucei* homologs. Identical residues are shown in black boxes. The amino acid residues involved in conserved domains are underlined. * indicates 10 amino acid intervals.

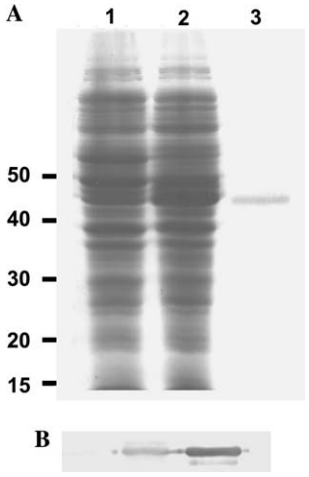

Fig. 3. Purification of the TcZFP1 recombinant protein. (A) SDS–PAGE analysis in 13% polyacrylamide gel of *E. coli* M15 extracts from uninduced (1), IPTG-induced (2 mM) cells (2), and purified recombinant protein (3). The molecular mass standards are indicated on the left (sizes in kDa). (B) *E. coli* M15 extracts from uninduced (1), IPTG-induced cells (2), and the purified GFP–tcZFP1 fusion protein (3) were reacted against an anti-His tag monoclonal antibody in a immunoblot. The His_6-tagged GFP–tcZFP fusion protein is clearly observed in lanes (2 and 3).

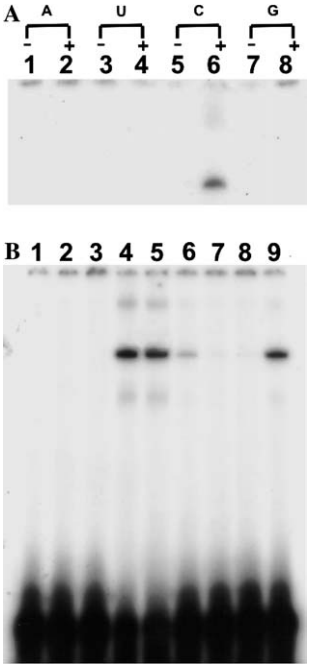

Fig. 4. Binding of the recombinant TcZFP1 protein to poly-homoribonucleotides. (A) Poly-homoribonucleotides were radiolabeled and used as a probe for EMSA. The runs of free radiolabeled probes are indicated in lanes 1, 3, 5, and 7. Binding reactions with 500 ng of the recombinant tcZFP1 and 0.5 ng of the respective labeled probe (approx. 10,000 cpm) are shown in lanes 2, 4, 6, and 8. (B) Specificity assays for the poly-C-ribonucleotide. Lane 1: free radiolabeled probe; lanes 2 and 3: binding reactions with 100 and 500 ng of the recombinant GFP; lanes 4 and 5: 100 and 500 ng of the recombinant tcZFP1; lanes 6–8: the poly-C-ribonucleotide was added as an unlabeled competitor—in a 1-, 10- or 100-fold molar excess, respectively—and incubated at room temperature for 10 min prior to the addition of the labeled probe; and lane 9: the ARE oligonucleotide was used as a competitor in a 100-fold molar excess. The gels were pre-run at 200 V at 4 °C and then run with the samples for 3–4 h at 250 V at 4 °C. Next, they were dried and used to expose X-ray films at −80 °C.

Meanwhile, the complex was not affected when a U-rich oligoribonucleotide (ARE) was used as a competitor (Fig. 4B). The complex was not significantly displaced by poly-A, poly-G or poly-U (data not shown), confirming the specificity of the recognition. The GFP recombinant protein was used as a control. These experiments clearly show that tcZFP1 presents selectivity for the target.

Binding of TcZFP1 to U-rich sequences

As it has been suggested that tbZFP1 could be involved in the regulation of mRNA stability by interacting with ARE elements [25], we used EMSA to test the ability of the recombinant tcZFP1 protein to bind ARE elements and different U-rich sequences. In our experimental conditions, no binding was detected with the ARE probe, whereas a specific complex was observed with the CU probe. A weak signal was also observed when the GU probe was used (Fig. 5A).

In *T. brucei*, a U-rich 26mer has been identified in the 3′-UTR of the EP/GPEET gene cluster mRNA [11]. This element is involved in the regulation of mRNA stability. Moreover, the HuR protein, which stabilizes ARE-containing mRNAs from mammalian cells [40], binds this sequence in vivo [20]. We also used the U-rich 26mer as a probe in EMSA. A strong binding of tcZFP1 was observed with this probe (Fig. 5A). The specificity of the binding was also tested using different probes as competitors. Interestingly, even a 100-fold excess of the ARE sequence was unable to compete (Fig. 5B). Meanwhile, the complex was readily displaced when competed with an equal amount of free probe.

Characterization of TcZFP1 complexes

To evaluate the relevance of the interaction between tcZFP1 and the above-mentioned nucleic probes, we investigated the binding affinity. The apparent dissociation constants (K_d) of the ribonucleoprotein complexes of the recombinant tcZFP1 protein with the poly-C and 26mer probe were determined by EMSA. Increasing amounts of the recombinant protein were used and equal amounts of the probe were loaded in each lane. The apparent K_d for each binding reaction was estimated as the protein concentration at which 50% of the probe was bound in the complex. The recombinant tcZFP1 recognizes poly-C with an apparent K_d of about 500 nM (Figs. 6A and B). This result is concordant with reported data for genome regulatory proteins. Nucleic acid-binding proteins containing RRM motifs and

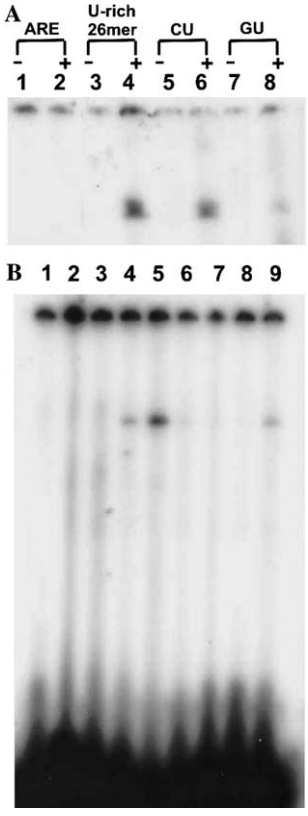

Fig. 5. Binding of the recombinant TcZFP1 protein to U-rich ribonucleotides. (A) U-rich ribonucleotides were radiolabeled and used as a probe for EMSA. The runs of free radiolabeled probes are indicated in lanes 1, 3, 5, and 7. Binding reactions with 500 ng of the recombinant tcZFP1 and 0.5 ng of the respective labeled probe (approx. 10,000 cpm) are shown in lanes 2, 4, 6, and 8. (B) Specificity assays for the U-rich 26mer oligoribonucleotide. Lane 1: free radiolabeled probe; lanes 2 and 3: binding reactions with 100 and 500 ng of the recombinant GFP; lanes 4 and 5: 100 and 500 ng of the recombinant tcZFP1; lanes 6–8: the U-rich 26mer oligoribonucleotide was added as an unlabeled competitor—in a 1-, 10- or 100-fold molar excess, respectively—and incubated at room temperature for 10 min prior to the addition of the labeled probe; and lane 9: the ARE oligonucleotide was used as a competitor in a 100-fold molar excess.

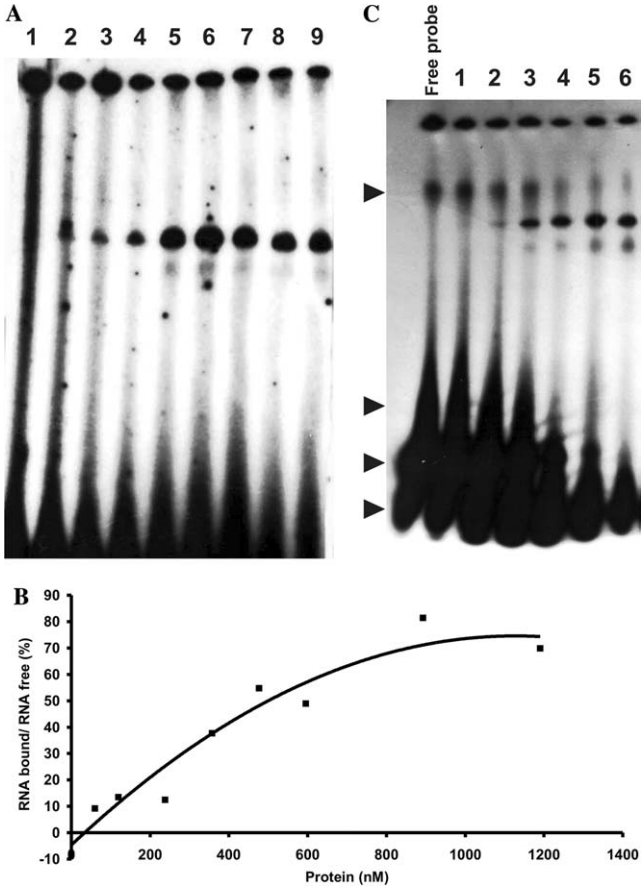

Fig. 6. Affinity analysis of the complexes formed by the recombinant TcZFP1 and the poly-C and U-rich 26mer oligoribonucleotides. (A) Binding reactions with the poly-C probe were carried out using increasing amounts of the recombinant tcZFP1 protein and 0.5 ng of the labeled probe (approx. 10,000 cpm). Lanes 1–9: binding reactions with 10, 50, 100, 200, 300, 400, 500, 750, and 1000 ng of protein, respectively. (B) To estimate the apparent K_d, quantitative data from (A) were plotted as the percentage of bound to free probe against tcZFP1 concentration (nM). (C) Binding reactions with the U-rich 26mer oligoribonucleotide were carried out as in (A). Lanes 1–6: binding reactions with 10, 50, 100, 200, 500, and 1000 ng of protein, respectively.

showing similar K_d values have recently been reported in *T. cruzi* [24].

Although tcZFP1 complexes were clearly formed at low protein concentrations with both the poly-C and the 26mer probes (50 and 100 ng, respectively), quantitative-binding affinity analysis revealed significant differences between the two probes. The K_d of the recombinant protein with the U-rich 26mer could not be unequivocally determined because of the presence of several forms of the free probe (indicated by arrowheads in the figure) which may exhibit different affinities for tcZFP1 (Fig. 6C). The observed multiple bands, most likely different conformers, could render misleading quantitative data.

To elucidate the characteristics of tcZFP1 complexes, we analyzed kinetic-binding parameters. EMSA run-off experiments were performed to study the stability of the complexes. The tcZFP1 complex with the poly-C probe showed a half-life of about 12 min (Figs. 7A and B). The half-life for the tcZFP1–26mer complex could not be estimated because the complex dissociated in less than 1 min (Fig. 7C). Although both complexes formed at similar low protein concentrations, the tcZFP1–26mer complex is much less stable than that formed by tcZFP1 and the poly-C probe.

Discussion

Gene expression in *T. cruzi* is fundamentally regulated by post-transcriptional mechanisms involving the control of mRNA stability and translation. *T. cruzi* also undergoes differentiation. Hence, in addition to the general mRNA decay machinery, *T. cruzi* must have

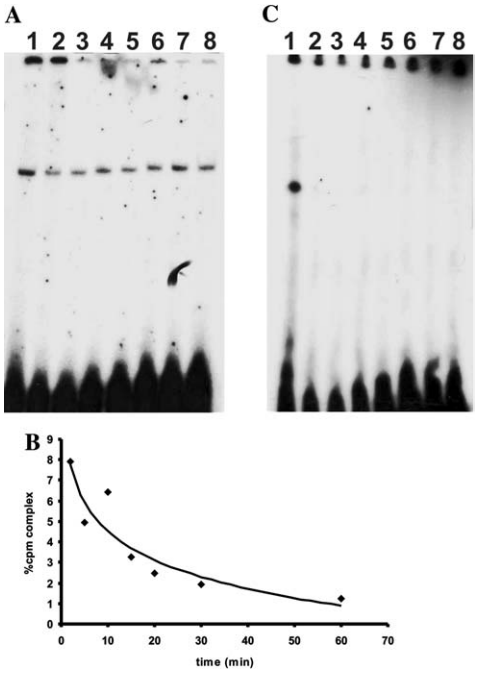

Fig. 7. Analysis of the stability of complexes formed by the recombinant TcZFP1 and the poly-C and U-rich 26mer oligoribonucleotides. (A) Binding reactions were carried out using 500 ng of the recombinant tcZFP1 protein and 0.5 ng of the poly-C-labeled probe. After complex formation, a 1000-fold molar excess of the unlabeled probe was added. Lanes 1–8: aliquots were removed and run on a gel to determine the amount of remaining complex at 0, 2, 5, 10, 15, 20, 30, and 60 min, respectively. (B) To estimate the dissociation complex half-life, the amount of remaining labeled complex (cpm normalized by total cpm in the lane), as determined in (A), was plotted against time. (C) Binding reactions with the U-rich 26mer oligoribonucleotide were carried out as in (A). Lanes 1–8: 0, 2, 5, 10, 15, 20, 30, and 60 min, respectively. The electrophoretic migration of the free probe and putative conformers is indicated in the figure.

stage-specific mRNA decay pathways to allow it to modulate its gene expression pattern rapidly according to the different environments encountered during its life cycle. The stability of the mRNA depends on interactions between *cis*-acting elements within the mRNA and *trans*-acting factors. It is reasonable to assume that parasite differentiation involves dynamic interactions between different proteins and the basal decay machinery to establish the stage-specific mRNA decay pathways. Hence, the identification of genes that encode products involved in mRNA decay is essential to determine how the parasite survives in different hosts and acquires its infectiveness by modulating its gene expression.

We cloned two genes encoding *T. cruzi* zinc finger proteins named tcZFP1 and tcZFP2. These proteins are homologous to tbZFP1 and tbZFP2, which are implicated in the regulation of the morphogenesis and differentiation of *T. brucei* [25]. These proteins share the unusual zinc finger motif (CCCH) that is characteristic of some RNA-binding proteins. CCCH zinc finger proteins can exert different functions, such as ensuring RNA stability, distribution or translation [27,29], and RNA processing [26,41]. However, little is known about the *cis*-acting elements within the RNA molecules involved in the putative interaction with CCCH zinc finger proteins in trypanosomatids. For this reason, we used EMSA to define the potential sequence targets of tcZFP1 in vitro.

As a first approach, we investigated the interaction of tcZFP1 with poly homoribonucleotides. EMSA showed that tcZFP1 binds specifically to poly-C oligoribonucleotides. This raises the possibility that tcZFP1 exerts its role by interacting with C-rich sequences in *T. cruzi* mRNAs. Poly-C-binding proteins play a role in both mRNA stability and translational regulation in eukaryotes. The most striking examples are the αCP1 and αCP2 proteins, which recognize a C-rich region in the 3′-UTR of the mammalian α-globin mRNA and form a protein complex with the poly (A)-binding protein that regulates the erythrocyte-specific accumulation of this mRNA [42–44].

We then investigated the interaction of tcZFP1 with ARE-like sequences, as tristetrapolin, a mammalian CCCH zinc finger protein, plays an important role in processes such as cell growth, differentiation, and immune response by destabilizing specific ARE-containing mRNAs [28,31]. In addition, ARE-like sequences within the 3′-UTR of trypanosomatid mRNAs have been shown to target mRNAs for stage-specific decay [3,45]. It has been suggested that tbZFP1 can also bind this element [25]. However, our results showed that the recombinant tcZFP1 does not bind to the ARE sequence in vitro. Surprisingly, binding was observed with other U-rich sequences. These results were not expected as no binding was observed with the poly-U probe. Nevertheless, the biological meaning of these interactions is doubtful due to the instability of the complex.

We analyzed the relevance of the specific complex formed between tcZFP1 and the poly-C ribopolymer with regard to stability and affinity. The parameters determined in our conditions must be considered as relative as physicochemical parameters are only accurate in the presence of pure protein. In our experimental conditions, we estimate that the apparent K_d was about 500 nM for the tcZFP1 fusion protein. This clearly shows that tcZFP1 shows similar selectivity and affinity to nucleic acid-binding regulatory proteins. A similar approach recently showed that *T. cruzi* RNA-binding proteins containing RRM motifs have similar K_d values [23,24].

It is possible that tcZFP1 regulates the half-lives of trypanosome mRNAs in several ways. For example, protein–protein interactions might create dynamic complexes that stabilize or destabilize mRNAs in a stage-specific manner or even control mRNA translation. Accordingly, it has been shown that AUF1/

hnRNP-D, a protein implicated in ARE-mediated mRNA decay, can interact with the poly-C-binding protein αCP1 in the α-globin mRNA α-complex. This suggests that the α-complex is dynamic and is involved in both mRNA stabilization and mRNA decay [46].

The role of CCCH zinc finger proteins in trypanosome homeostasis and differentiation remains unknown. In addition to their role in mRNA stability, it is possible that these proteins also play a role in polycistronic mRNA processing. Indeed, a new CCCH zinc finger protein, named tbCPSF30, has been shown to be a component of the cleavage and polyadenylation machinery of *T. brucei* [41].

It remains unclear whether tcZFP1 modulates stage-specific mRNA decay or the accessibility of mRNAs to the translational machinery. Interestingly, the tcZFP1 mRNA is found to be more abundant in the polysomal mRNA population of stressed parasites when compared with epimastigotes and differentiating epimastigotes (cells adherent after 24 h) from the in vitro metacyclogenesis. Since polysomal mRNA is engaged in translation, these data indicate that the amount of tcZFP1 is also modulated during *T. cruzi* differentiation. The same is observed for tbZFP1 which is transiently enriched during *T. brucei* differentiation from bloodstream to procyclic form parasites [25].

We are currently carrying out gene knockout and overexpression experiments in conjunction with microarray analysis to provide new insights into the mRNA targets of these proteins, as by changing the tcZFP1 and tcZFP2 levels we might be able to detect their target mRNAs due to changes in their half-lives and translation.

Acknowledgments

We thank Dr. Keith Mathews for providing data about *T. brucei* ZFP1 and for his interest in this investigation. This work received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (PRONEX, PROSUL). B.D. has a fellowship from Fiocruz-CNPq, A.C.S.U., C.M.P., M.A.K., and S.G. acknowledge CNPq for their fellowships, and G.F.A.P. acknowledges CAPES for her fellowship.

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