TcUBP-1, an mRNA Destabilizing Factor from Trypanosomes, Homodimerizes and Interacts with Novel AU-rich Element- and Poly(A)-binding Proteins Forming a Ribonucleoprotein Complex*

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Trypanosomes, protozoan parasites causing worldwide infections in human and animals, mostly regulate protein expression through post-transcriptional mechanisms and not at the transcription initiation level. We have previously identified a Trypanosoma cruzi RNA-binding protein named TcUBP-1. This protein is involved in mRNA destabilization in vitro through binding to AU-rich elements in the 3′-untranslated region of SMUG mucin mRNAs (D’Orso, I., and Frasch, A. C. (2001) J. Biol. Chem. 276, 34801–34809). In this work we show that TcUBP-1 is part of an ~450-kDa ribonucleoprotein complex with a poly(A)-binding protein and a novel 18-kDa RNA-binding protein, named TcUBP-2. Recombinant TcUBP-1 and TcUBP-2 proteins recognize U-rich RNAs with similar specificity and affinity through the ~92-amino acid RNA recognition motif. TcUBPs can homo- and heterodimerize in vitro through the glycine-rich C-terminal region. This interaction was also detected in vivo by co-immunoprecipitation of the ribonucleoprotein complex and using yeast two-hybrid assay. The poly(A)-binding protein identified was shown to disrupt the formation of TcUBP-1, but not TcUBP-2, homodimers in vitro. The possible role of TcUBP-1 ligands in the pathways that govern mRNA-stability and stage-specific expression in trypanosomes is discussed.

Trypanosoma cruzi is the etiological agent of Chagas’ disease, an endemic illness in Latin America. The parasite has a life cycle that includes a vertebrate and an insect vector, with different developmental stages involved in each host. In the insect, two main forms of the parasite are present: replicative epimastigotes and metacyclic trypomastigotes. The latter form is infective to humans after being released on the skin or mucosa with the feces of the bug. Metacyclic trypomastigotes invade host cells and differentiate into a replicative amastigote form that differentiates into bloodstream trypanomastigotes. The latter stage is able to invade a wide variety of cells, thus propagating the infection. The cycle closes when the hematophagous vector ingests circulating trypanomastigotes with its blood meal. Trypanosomes, protozoan parasites of the order Kinetoplastida, have particular features in terms of mechanisms leading to protein expression. RNA polymerase I promoters were identified in rDNA but also in genes encoding the variable surface antigens from African trypanosomes (1). Conversely, only few RNA polymerase II promoters were described (2). As part of the maturation process, a common 39-nucleotide RNA, named spliced leader, is added to all trypanosome mRNAs by a trans-splicing process. This phenomenon was shown to be couple to 3′-poly(A) tail addition during polycistrionic RNA processing (3). As a consequence, and at variance with higher eukaryotic cells, the control of protein expression in trypanosomatids is mainly post-transcriptional (4). However, little information is available on the relative importance of these processes and how they operate jointly in the parasite.

One of the possible mechanisms to regulate protein expression is through modification of the half-life of mRNAs. Several cis-elements located throughout the mRNA, coding and/or untranslated regions (UTRs),1 were identified (5–7). However, only few of them were found to be recognized specifically by trans-acting factors (8). We have found previously (9) two distinct cis-elements located in the 3′ cis-elements of SMUG transcripts in the non-infective replicative trypanomastigote stage of the parasite. Conversely, a 27-nucleotide G-rich cis-element stabilizes SMUG mRNAs in the non-infective replicative epimastigote stage of the parasite (8). These results suggest that both elements, ARE and the G-rich cis-element, act coordinate in a developmentally regulated manner and are recognized by specific RNA-binding proteins (8). Recently, we described that this ARE sequence was recognized by a single RRM-type RNA-binding protein named TcUBP-1, for T. cruzi Uridine-binding protein. In vivo, TcUBP-1 was shown to destabilize SMUG mRNAs in the epimastigote stage of the parasite (10). Furthermore, and at variance with what occurs in yeast, the processes of 3′-5′ and 5′-3′ exonucleolytic cleavage were shown to be active in trypanosomes (11). The identification of an exosome in T. brucei (12) suggests that an early mechanism of mRNA maturation, mediated by 3′-5′ exonucleases involved...
GST fusion proteins were cleaved with thrombin (Sigma). The recombinant proteins were purified using GST-agarose columns (Sigma). Annealing at the N-terminal of the cDNAs and partial deletions of both were amplified by RT-PCR (see Table I). They were cloned into the pGEX-2T vector (Amersham Biosciences). Keyhole limpet hemocyanin-conjugated peptides were injected into rabbits and mice with Freund’s adjuvant three times at 2-week intervals. For Western blot analysis, samples were fractionated on SDS-PAGE gels transferred to Hybond®NC nitrocellulose (Amer sham Biosciences), probed with primary antibodies, and developed using horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies and the Supersignal® West Pico chemiluminescent substrate (Pierce), according to manufacturer’s instructions. Antibody Production and Western Blot Analysis—Cytosolic protein extracts and subcellular fractionation were done as described (8). Immunoprecipitations—A cytosolic extract corresponding to 10° parasites or gel filtration chromatography column fractions were incubated with mouse pre-immune serum, anti-TcUBP-1, anti-TcUBP-2, or anti-TcPABP1 polyclonal antibodies for 2 h at 4 °C with gentle mixing. A 50% slurry of protein A-Sepharose was added to the mixture and incubated for 2 h more. The mixture was centrifuged at 1000 x g for 1 min, and the resin was washed four times with 300 μl of Tris-buffered saline–Tween 0.05%. Proteins were eluted with 2× Laemmli buffer.

### EXPERIMENTAL PROCEDURES

**Parasite Cultures**—T. cruzi CL-Brener cloned stock (14) was used. Different stages of the parasite were obtained as described previously (15).

**Cloning of Tcubp-1 and Tcubp-2 Domain—**During the cloning of Tcubp-1 by RT-PCR using RNP-1 oligonucleotide (5’-aaacctacatcaataagggc-3’), a second clone, named Tcubp-2, was identified. Another RT-PCR was done with primer oligo(dT)18-anchor (5’-gggcttgcggcggctg(18)-3’). Second strand DNA synthesis was done with anchor and with a primer annealing at the N-terminal of Tcubp-2, named NH2-sense (5’-atgtctcaacagatgcaatac-3’). These products were cloned in pGEM-T Easy (Promega) and sequenced in an ABI Prism 373 sequencer. The sequence reported here has been submitted to GenBank™ with accession number AF497746.

**Recombinant TcUBP Proteins Expression and Purification—**Tcubp-1 and Tcubp-2 cDNAs and partial deletions of both were amplified by PCR (see Table I). They were cloned into the BamHI and EcoRI restriction endonuclease sites of the pGEX-2T vector (Amer sham Biosciences), generating a glutathione S-transferase (GST) fusion and transformed in Esherichia coli strain BL21 DE3 pLysS (Novagen). Cultures were induced with isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Recombinant proteins were purified using GST-agarose columns (Sigma). GST fusion proteins were cleaved with thrombin (Sigma). The reactions were performed with 1 unit of thrombin/100 μg of protein in a buffer containing 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 2.5 mM CaCl2 during 2 h at 25 °C.

**Antibody Production and Western Blot Analysis—**An antibody against Tcubp-1 RRM was prepared as described (10). Two specific peptides of Tcubp-1, VSQTPDYGQTAC, and Tcubp-2, RNRNGYST-FGAC, were synthesized (Sigma GENOSYS). The C-terminal cysteine residues were added to conjugate the peptides with maleimide-activated keyhole limpet hemocyanin according to the manufacturer’s instructions (Pierce). Keyhole limpet hemocyanin-conjugated peptides were injected into rabbits and mice with Freund’s adjuvant three times at 2-week intervals. For Western blot analysis, samples were fractionated on SDS-PAGE gels transferred to Hybond®NC nitrocellulose (Amer sham Biosciences), probed with primary antibodies, and developed using horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies and the Supersignal® West Pico chemiluminescent substrate (Pierce), according to manufacturer’s instructions.

**Cloning of Tcubp-1 and Tcubp-2 Domain—**A 249-bp fragment corresponding to region 1 (16) was amplified by PCR with oligonucleotides PABC-1 (5’-gtatcttggcttcaggggaccacg-3’) and PABC-2 (5’-gtatcttggcttcaggggaccacg-3’) and was named Tcabc (introduced EcoRI and BamHI sites are underlined). Tcubp-1 was amplified by PCR with primer PABP-N (5’-gaattctctgttacgggttcgcagcg-3’) and PABC-2. Both products were cloned into pGEX-2T vector (Amer sham Biosciences) and induced as indicated above. An antibody against TcPABC was raised and named anti-TcPABP1.

**Protein Extract Preparation—**Cytosolic protein extracts and subcellular fractionation were done as described (8).

**Immunoprecipitations—**A cytosolic extract corresponding to 10° parasites or gel filtration chromatography column fractions were incubated with mouse pre-immune serum, anti-TcUBP-1, anti-TcUBP-2, or anti-TcPABP1 polyclonal antibodies for 2 h at 4 °C with gentle mixing. A 50% slurry of protein A-Sepharose was added to the mixture and incubated for 2 h more. The mixture was centrifuged at 1000 × g for 1 min, and the resin was washed four times with 300 μl of Tris-buffered saline–Tween 0.05%. Proteins were eluted with 2× Laemmli buffer.

**Gel Filtration Chromatography—**Gel filtration was carried out with 300 μl of an epimastigote protein extract (10 mg/ml) on a Bio-Sil SEC 250 column (Bio-Rad). A Superdex 75 HR 10/30 (Amer sham Biosciences) was used for the determination of apparent molecular masses of the recombinant proteins. Columns were equilibrated in 20 mM Tris-HCl, pH 6.8, and 150 mM NaCl. Fractions of 500 μl were collected.

**Cross-linking Studies—**Tcubp-1, Tcubp-2, and the deletion mutant proteins (Table I) were treated at room temperature with glutaraldehyde at a final concentration of 0.01% as indicated (17). The products were run on a SDS-PAGE and stained with Coomassie Blue.

**Dihydrazide-agarose RNA Cross-linking—**Homoribopolymers (rA, rC, rG, rU) were oxidized with NaIO4 and cross-linked to adipic acid dihydrazide-agarose beads (Sigma) as indicated previously (18). Purified proteins or trypanosome extracts were incubated for 1 h with RNA cross-linked beads and washed. Protein elution was done with 2× Laemmli buffer.

**In Vitro Protein Binding Experiments—**GST pull-down was carried out with 5 μg of GST or GST fusion proteins immobilized on glutathione beads (Sigma) and 5 μg of Tcubp-1 or Tcubp-2, without GST tag. Columns were equilibrated with Tris-buffered saline, and after washing with Tris-buffered saline–Triton 0.1%, eluted proteins were loaded onto a SDS-PAGE and stained with Coomassie Blue.

**In Vitro Transcription—**RNA production was done according to pre-
RESULTS

Tcubp-2 Encodes a Single RRM-type RNA-binding Protein—During Tcubp-1 cloning (10), we also identified, by RT-PCR, a product and enables the cells to grow on minimal medium lacking uracil (-Galactosidase assays were carried out as described previously (19). TcUBP-1 and TcUBP-2, two RRM-type RNA-binding proteins derived from cDNA sequence. The alignment was done using ClustalW (20). Gly- and Gln-rich regions and the RNP-2 and RNP-1 sequences within the RRM motif are indicated. The predicted α and β secondary structures are shown as determined (www.embl-heidelberg.de/predictprotein). B, scheme of TcUBP-1 and TcUBP-2 proteins showing the RRM, VR, Gly-rich region (GLY), and Gln-rich region (GLN). 

Tcubp-2 expression levels were analyzed by Western blot, using total parasite extracts from the different stages of the parasite. TcUBP-2 was detected preferentially in epimastigotes (Fig. 2D), showing that it is expressed differentially during parasite development. As control of this experiment, the same
A Ribonucleoprotein Complex Containing TcUBP-2 and the Destabilizing Factor TcUBP-1—Because TcUBP-1 was shown to be involved in post-transcriptional regulation (10), it might exert its effect through protein-protein interactions. Thus, we asked whether TcUBP-1 forms a protein complex in vivo in the epimastigote stage. Gel filtration experiments were performed with cell-free extracts pre-treated with Buffer A (150 mM NaCl) and Buffer B (150 mM NaCl and -0.1 µg/µl RNase A) to a 20 mM Tris-HCl, pH 6.8, buffer. The different extracts were applied to a BioSil SEC250 gel chromatography column, and fractions of 0.5 ml were collected. They are indicated as Gel Chromatography Fractions above each panel. Samples were then analyzed by Western blot with an anti-RRM antibody. The position of TcUBP-1, TcUBP-1m, and TcUBP-2 protein is indicated with arrows. Ext, refers to the protein extract before applying to the column. In fractions 1 to 7, no proteins cross-reacting with the antibody were detected (not shown). The molecular mass markers used in the Gel Chromatography column are indicated above each panel as follows: 670 kDa, tyroglobulin; 180 kDa, a2-macroglobulin; 45 kDa, ovalbumin; 17 kDa, myoglobin.

A Ribonucleoprotein Complex Containing TcUBP-2 and the Destabilizing Factor TcUBP-1 — Because TcUBP-1 was shown to be involved in post-transcriptional regulation (10), it might exert its effect through protein-protein interactions. Thus, we asked whether TcUBP-1 forms a protein complex in vivo in the epimastigote stage. Gel filtration experiments were performed with cell-free extracts pre-treated with Buffer A (150 mM NaCl) and Buffer B (150 mM NaCl and -0.1 µg/µl RNase A). After pre-treatment of the extracts for 30 min, they were applied on a Bio-Sil SEC 250 column, and the eluates were analyzed by Western blot using an anti-RRM antibody. The position of TcUBP-1, TcUBP-1m, and TcUBP-2 protein is indicated with arrows. Ext, refers to the protein extract before applying to the column. In fractions 1 to 7, no proteins cross-reacting with the antibody were detected (not shown). The molecular mass markers used in the Gel Chromatography column are indicated above each panel as follows: 670 kDa, tyroglobulin; 180 kDa, a2-macroglobulin; 45 kDa, ovalbumin; 17 kDa, myoglobin.

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mRNAs (21). Because TcUBP-1 binds to SMUG 3’UTR and regulates its mRNA stability levels, we asked whether TcPABP1 might be present in this complex. An immunoprecipitation was carried out with anti-TcUBP-1, anti-TcUBP-2, or anti-TcPABP1 antibodies followed by a Western blot using the anti-TcPABP1 antibody. We found evidence that the ribonucleoprotein complex containing the heterodimer also contains TcPABP1 (Fig. 4A). Although two different bands of 55 and 66 kDa were detected, the 66-kDa band was described as being TcPABP1 (16).

**TeCuBP-1 and TeCuBP-2 Interact in Vivo in a Two-hybrid System**—A two-hybrid assay was performed to corroborate TcUBP-1 homo- and heterodimerization with TcUBP-2 in a heterologous system. The coding sequence of TcUBP-1 was fused to the LexA-DBD in the plasmid pBTM116 to serve as bait (19). Along with the LexA-DBD-TcUBP-1, we used the TcUBP-2 homolog to test the interaction. We found that LexA-DBD-TcUBP-1 and TcUBP-2 cDNAs were cloned into the pVP16 fused to VP16 activation domain. We test this in binding assay with LexA-DBD-TcUBP-1. The co-transformation of L40 yeast strain with LexA-TcUBP-1 and VP16-TcUBP-1 or VP16-TcUBP-2 allowed the growth of transformants on medium without histidine and selection for GAL4-dependent lacZ activity (Table II).

**Comparison between the $K_d$ of TcUBP-1 and TcUBP-2**

| RNA   | TcUBP-1 | TcUBP-2 |
|-------|---------|---------|
| S     | 500 ± 25 | 250 ± 10 |
| P1    | 150 ± 5  | 100 ± 5  |
| P2    | 750 ± 50 | 750 ± 65 |
| P3    | >1000    | 850 ± 55 |
| P1-GGG| 250 ± 15 | 200 ± 15 |

**Interaction between TcUBP Proteins and TcPABP1 in Different Stages of Differentiation**—We next analyzed the interaction of TcUBPs and TcPABP1 throughout parasite development. Total trypanosome extracts were prepared from the different parasite stages, incubated with anti-TcUBP-1 antibody for immunoprecipitation, followed by Western blot with anti-TcPABP1. This experiment demonstrated that TcUBP-1 interacts with TcPABP1 in the epimastigote and trypomastigote stages (Fig. 4C). Conversely, TcUBP-2 co-precipitates with TcPABP1 in the epimastigote stage (Fig. 4B) and not in other parasite stages (not shown). Because the complex containing TcUBPs in epimastigotes was disrupted by RNase A treatment (Fig. 3B), we asked whether the interaction between TcUBP-1 and TcUBP-2 in this stage was dependent on RNA. Immunoprecipitations were done with anti-TcUBP-1 antibody using cell-free extracts pre-treated or not for 30 min with RNase A. When the extract was RNase-treated there was no detectable TcPABP1 in the immunoprecipitate (Fig. 4D). Conversely, in the trypomastigote stage the interaction was not dependent on RNA, showing that TcUBP-1 and TcPABP1 make direct protein-protein contact.

**TcUBP-1 and TcUBP-2 Present the Same Binding Affinity and Specificity to Homoribopolymers and U-rich RNA**—The binding specificity of TcUBP-2 was tested and compared with TcUBP-1. An electrophoresis mobility shift assay (EMSA) was performed with a labeled U-rich element named P1, in the presence of different amounts of each of the four homoribopolymers (Fig. 5B). We found that TcUBP-1 and TcUBP-2 RNA-binding activity was competed in a concentration-dependent manner with poly(U) and not by poly(A) or poly(C). In addition, it can be competed at high concentrations (500X) of poly(G). These results confirmed that recombinant proteins recognize poly(U) RNA, as do the native ones (see Fig. 2E).

**Different U-rich RNAs were used to determine by EMSA the apparent dissociation constants ($K_d$) of the ribonucleoprotein complexes formed by TcUBP-2 and TcUBP-1 (Fig. 5). The apparent $K_d$ for each reaction was calculated by determining the protein concentration at which 50% of the RNA was bound. TcUBP-2 recognized P1 RNA with an apparent $K_d$ of ~100 nM, and TcUBP-1 recognized it with a $K_d$ of ~150 nM. A new RNA, which has three uridine to guanosine changes in the last U-rich stretch of P1, was made and named P1-GGG (see Fig. 5A). These changes had a slight effect on TcUBP-2 $K_d$ (~200 nM) and TcUBP-1 $K_d$ (~250 nM). As it was described with TcUBP-1 (10), the S RNA (Fig. 5) is bound by TcUBP-2 with a slightly high $K_d$ (~250 nM). Conversely, P2 and P3 RNAs (Fig. 5), which...
have shorter U-rich stretches, were recognized by TcUBP-2 but with lower affinities ($K_d$ values of ~750 and ~850 nM, respectively) (Table II). These results suggest that U-rich stretches are the most important component of the RNA recognized by TcUBPs proteins. Also, GU-rich RNAs are better recognized than AU-rich sequences, and the RNA-binding activity of TcUBP-1 was clearly similar to TcUBP-2 (Fig. 5, C and D). A summary of TcUBPs RNA-binding activities is shown in Table II. This comparison reflects that TcUBP-1 and TcUBP-2 have similar $K_d$ values to the U-rich RNAs tested in this work.

Mapping of the Minimal Region Required for RNA Binding in TcUBPs Proteins—We demonstrated that TcUBP-1 and TcUBP-2 recognized the P1 RNA with similar apparent $K_d$ values (Fig. 5). These results suggest that the differences in the primary sequence between both proteins might not contribute to RNA recognition and/or modulation of the RNA-binding activity. In addition, TcUBP-1 and TcUBP-2 dimerize when binding to RNA containing U-rich regions, and therefore, we hypothesize that the auxiliary domains might be crucial for their function in protein-protein interaction. To verify this hypothesis, several deletion mutants were constructed by PCR (Table I). The different proteins were named TcUBP-1AN, lacking the N-terminal region; TcUBP-1AQ, lacking the glutamine-rich region; TcUBP-1ANQ, lacking its N- and glutamine-rich regions; and two other constructs named TcUBP-1AQG1 and TcUBP-1AQG2 (Fig. 6A). The difference between these two last deletion mutants resides in their VR. The first one has this portion, composed by 14 extra amino acids (PGIAGAVGDGNGLY), after the predicted B4 sheet (Fig. 6A). However, both lack the motif GAYGGYGAY within the glycine-rich region. Similarly, TcUBP-2 deletions were TcUBP-2AN, lacking the N-terminal region; TcUBP-2AC, which lacks the C-terminal region; and TcUBP-2ACG1 and TcUBP-2ACG2 (Fig. 6C). The difference between these two last proteins is that the first one presents the VR region, composed by nine extra amino acids (VRNGVSTGF), in the C-terminal region.

The predicted secondary structure and heteronuclear-nuclear Overhauser effect NMR spectra of TcUBPs suggest that the auxiliary domains behave as random-coil, so none of these deletions should have any effect on TcUBP-1 and TcUBP-2 final folding. The apparent $K_d$ values of all TcUBPs constructions were determined by EMSA. The majority of them present similar $K_d$ values of ~100–200 nM (Fig. 6A, B and D). However, TcUBP-1AQG2 showed a $K_d$ ~1000 nM (Fig. 6B), showing that the RNA affinity of this mutant is decreased more than 5-fold. Conversely, TcUBP-1AQG1 that contains the VR region at the C-terminal of the RRM motif showed the same $K_d$ as the complete TcUBP-1 protein. Similarly, all TcUBP-2 mutants, except for TcUBP-2ACG2, interact with RNA with the same affinity as the wild type TcUBP-2 protein (Fig. 6D). These experiments demonstrate that the ~92-amino acid RRM motif is the minimal region required for RNA binding in TcUBPs proteins. The function of C-terminal VR extension in the RRM motif require further investigation. It might be homologous to that of the RRM C-terminal extension present in RNA-binding proteins from other cell types (see “Discussion”).

The Glycine-rich Region of the Auxiliary Domain Is Critical for Dimerization—A decrease in the mobility of the ribonucleoprotein complex formed in the presence of protein amounts greater than 500 nM was observed with TcUBP-1 and all mutants proteins except for TcUBP-1AQG1 and TcUBP-1AQG2 (Fig. 6). Both proteins lack the glycine-rich region. Thus, these results suggest that this region might be important for dimerization. To study the formation of dimers in vitro, we performed cross-linking reactions with glutaraldehyde at a final concentration of 0.01% (Fig. 7). This is a zero-length cross-linker reagent that was described to form covalent bonds between proteins that are in contact (17). For this purpose, different TcUBP-1 mutant proteins listed in Fig. 7A were analyzed. Dimers were detected at concentrations greater than 500 nM (not shown). TcUBP-1AQ protein, which presents the VR- and glycine-rich regions within the C-terminal, was cross-linked efficiently (Fig. 7B, lane 4). The percentage of cross-linking in this protein was about 10–15%. Similar values of cross-linking were obtained with proteins from other cell types (17). Conversely, TcUBP-1AQG1 mutant protein formed dimers much less efficiently than TcUBP-1AQ (Fig. 7B, compare lanes 4 and 6), and TcUBP-1AQG2 did not react with the cross-linker (Fig. 7B, lane 8). The formation of dimers in TcUBP-1 was hardly detectable with the amount of protein used in this assay (Fig. 7B, lane 5). It might be possible that its C-terminal glutamine-
The glycine-rich region is critical for dimerization of TcUBP proteins. A, scheme of TcUBP-1 and mutant proteins used for the cross-linking studies. The position of wild type GAYGGYGAY sequence is indicated above the TcUBP-1 scheme. B, the proteins of panel A (1 µl) were incubated (+) or not (-) with 0.01% glutaraldehyde. Samples were resolved by SDSPAGE and stained with Coomassie Blue. Position of monomer and dimer forms is indicated with arrows at the right of the panel. The asterisk (*) indicates the position of TcUBP-1 dimer. The position of each lane is indicated below the figure.

The Formation of TcUBP-1, but Not TcUBP-2, Homodimers Is Disrupted by TcPABP1 Interaction in Vitro—We have shown previously that TcPABP1 interacts in vivo with TcUBP proteins in both epimastigote and trypomastigote stages (Fig. 4). Thus, we asked whether this interaction is also detected in vitro. For these propose, TcPabp1 was cloned and expressed as a GST fusion protein, rendering GST-TcPABP1. To determine whether TcUBP-1 and TcUBP-2, without GST tag, interact in vitro with GST-TcPABP1, a GST pull-down assay was done. Under the same assay conditions, GST-TcPABP1 interacts with TcUBP-1 but failed to interact with TcUBP-2 (Fig. 8A), suggesting that the binding of TcUBP-1 to TcPABP1 is mediated by protein-protein interactions and not mediated by RNA (Fig. 8A). We also analyzed by EMSA the effect of adding increasing concentrations of TcPABP1 in TcUBP-1 and TcUBP-2 U-rich RNA-binding assays. To ensure complete RNA recognition, ∼2 µM TcUBP-1 and TcUBP-2 proteins were used. Although no observable effect was detected on TcUBP-2 RNA-binding, TcUBP-1-binding activity was modified by increasing concentrations of TcPABP1 (Fig. 8B). TcPABP1 did not displace TcUBP-1 protein from RNA, because no free RNA is detected at high amounts. Conversely, it disrupted or knocked out dimer formation on U-rich RNA, rendering a monomeric RNA recognition pattern in solution (Fig. 8B). TcUBP-1 interaction with TcPABP1 might involve N- or C-terminal regions of TcUBP-1, because these regions are different between TcUBP-1 and TcUBP-2.

DISCUSSION
In this work we have provided evidence for the existence of two very closely related RNA-binding proteins, TcUBP-1 and TcUBP-2, that present an identical RRM motif with distinct N- and C-terminal regions. The C-terminal region of TcUBP-1 can be divided into two parts, a short glycine-rich region and a large glutamine-rich random-coil domain. Conversely, TcUBP-2 presents a glycine-rich region larger and with three YGG motives (Fig. 1). TcUBP-1 presents affinity for U-rich elements in vitro and in vivo (10; and this work), and both proteins recognize with high affinity AU- and GU-rich elements. Our ongoing NMR structure of TcUBP-1 and TcUBP-2 suggest that they present a similar ββββββ topology. Thus, this might explain why both proteins present similar Kd values with the RNAs used for the EMSA experiments (see Table II). Although the RRM is almost identical, the C-terminal extension of this motif, named VR region, is different, and it might have a regulatory role. Deletion of the VR region produces an increase in the apparent Kd in both TcUBP-1 proteins (Fig. 6). Similarly, an NMR and biochemical study of U1A RRM motif but lacking its C-terminal extension showed that it has the same folding topology but does not bind RNA (22). Moreover, these residues were involved in structure stability of the free protein (23). In heterogeneous nuclear RNP-D, the C-terminal of RRM2 was important in conferring high affinity for ARE binding (24). This phenomenon suggests that these residues
localized in the VR region might be important in conferring structure stability by a possible interaction with residues in the β1β3 sheets that make contact with the RNA.

Of great importance is the modular RRM organization of RNA-binding proteins in higher eukaryote cell types. They present multiple RRM motifs, and its composition is what determines the final affinity for the RNA. The first RRM (RRM1) is the most important in terms of RNA affinity and specificity. However, the other RRM motifs have an accessory function, such as oligomerization and localization (25), structure stability in solution (23), and protein-protein interaction (26). Analysis of HuR deletion mutants allowed Park et al. (27) to suggest that RRM1 is critical for ARE binding but that at least one additional RRM is required to achieve a \( K_d \) in the nanomolar range. Both RRM1 and RRM2 are important for ARE affinity, even though they are not sufficient (24). It is important to conclude that certain RNA-binding proteins require only one domain to achieve RNA-binding specificity and affinity, such as U1A (28). Conversely, other proteins have been shown to require combinations of at least two (27) or occasionally more domains (29). Several common features have emerged from the comparison of two tandem RRM structures such as those in Sxl (30) and PABP1 (31). Each of these proteins contains short interdomain linkers of about 8–11 residues. These regions are highly mobile in the free protein making the two domains structurally independent (32).

Homodimerization, the common feature of prokaryotic transcription factors, is extended in eukaryotes by heterodimerization. This possibility increases the range of DNA sequences that can be recognized and the degree of binding specificity. In evolutionary terms, this increase in range is necessary when genomes become larger and more complex (33). We have provided here evidence demonstrating that the glycine-rich region of TcUBPs is important for dimerization (see Figs. 6 and 7). However, the fact that no dimers can be detected by cross-linking with TcUBP-1, at difference with TcUBP-1ΔQ (Fig. 7), suggests that its C-terminal region might regulate the accessibility and interaction with itself and/or other proteins. It was described that shorter coiled-coils of some transcription factors might either promote or prevent formation of homo- and/or heterodimers (33). Thus, it is likely that in the parasite both homo- and heterodimerization might be regulated processes. In this work we showed that TcUBPs, having a single RRM motif, bind RNA with high affinity and can dimerize by the glycine-rich flexible region. Thus, an even higher affinity might be achieved in vivo through homo- and heterodimerization of TcUBPs, allowing the simultaneous binding of two RRMs to the RNA. The glycine-rich region might contribute to the mobility and flexibility of the RRM motif in the dimer form, as deduced from our results and comparison with RRM-type proteins in other cell types. In general terms, the glycine-rich domains found in several RNA-binding proteins do not have any structural features beyond the likelihood that stretches of residues such as Gly or Pro result in flexible coils. This region was demonstrated to be involved in protein-protein interactions and to contribute to the RNA-binding free energy (34, 35).

TcUBP-1 and TcUBP-2 form part of a ribonucleoprotein complex containing TcPABP1. TcUBP-2 co-precipitates in the epimastigote stage, whereas TcUBP-1 precipitates with TcPABP1 in epimastigote and trypomastigote stages (Fig. 4). In vitro, TcUBP-1 interacts with TcPABP1, in the absence of RNA, and the effect of this interaction was to disrupt the formation of TcUBP-1, but not TcUBP-2, homodimers (Fig. 8). The domains involved in these interactions have not been identified. However, we speculate that the C terminus of TcUBP-1 might be involved, because this is the most dissimilar region when comparing with TcUBP-2. Although TcUBP-1 lacks the PABC consensus sequence described for PABP1 partners in human (36), this possibility must be tested further. In higher eukaryotes, when complexed to the poly(A)-tail, PABP1 circularizes mRNA molecules via its interaction with translational initiation factors, which results in mRNA stabilization (21). Recently, it has been shown that binding of some AU-rich-binding proteins to ARE may alter the interaction between PABP1 and the poly(A)-tail, thereby providing access to a poly(A)-ribonuclease (37). Rapid mRNA degradation involves the recognition of AREs by AU-rich-binding proteins, several of which have been shown to recruit the AREome and cause the reduction of PABP1 affinity for the poly(A)-tail (37). In our model (Fig. 9), the interaction of TcUBP-1 and TcPABP1 in vitro disrupts the formation of TcUBP-1 homodimers and might also change the affinity of TcPABP1 for the poly(A)-tail. The possibility that in trypomastigotes, the presence of TcUBP-1 protein without TcUBP-2 on SMUG 3’UTR may recruit the exosome or a poly(A)-ribonuclease activity (Fig. 9), is unlikely (21). We suggest that, in the epimastigote stage, the presence of TcUBP-2 and other yet unidentified factors might stabilize the interaction between TcUBP-1 and TcPABP1 within the ribonucleoprotein complex. This can prevent, at least in part, the disruption of TcUBP-1 homodimers (Fig. 8). Although our results provide evidence for the role of these ARE-binding proteins in the regulation of mRNA turnover in trypanosomes, establishing the mechanism by which TcUBPs stimulate mRNA stabilization/distabilization will require additional experiments.

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