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Research paper

A new antimicrobial protein from the anterior midgut of *Triatoma infestans* mediates *Trypanosoma cruzi* establishment by controlling the microbiota

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ABSTRACT

The Reduviid *Triatoma infestans* is a vector for the protozoan *Trypanosoma cruzi*, the etiological agent of Chagas disease. The parasite must address the defense molecules and microbiota that colonize the anterior midgut of *T. infestans*. To obtain insight into *T. cruzi* - microbiota interactions in triatomine insects, we characterized a new antimicrobial product from the anterior midgut of *T. infestans* (TiAP) that may be involved in these relationships. The TiAP DNA fragment was cloned and expressed in a bacterial system, and the effect of the protein on bacteria and *T. cruzi* was evaluated by RNAi, qPCR and antimicrobial experiments. The number of *T. cruzi* in *T. infestans* anterior midguts was significantly lower in TiAP knockdown insects than in unsilenced groups. We also verified that the amount of bacteria in silenced *T. infestans* is approximately 600-fold higher than in unsilenced insects by qPCR. The 327-bp cDNA fragment that encodes mature TiAP was cloned into the pET-14b vector and expressed fused to a His-tag in *Escherichia coli* C43. The recombinant protein (rTiAP) was purified using an Ni-NTA column, followed by a HiTrap SP column. According to a trypanocidal assay, rTiAP did not interfere with the viability of *T. cruzi* trypomastigotes. Moreover, in antimicrobial experiments using *E. coli* and *Micrococcus luteus*, the protein was only bacteriostatic for Gram-negative bacteria. The data indicate that infection by *T. cruzi* increases the expression of TiAP to modulate the microbiota. The inhibition of microbiota growth by TiAP is important for parasite establishment in the *T. infestans* anterior midgut.

1. Introduction

Triatoma infestans is an important vector of *Trypanosoma cruzi*, the etiological agent of Chagas disease in Latin America [1–3]. *T. cruzi* remains exclusively inside the insect gut, where the parasite counteracts several vector defense factors [4,5]. Relatively few studies have focused on the importance of immune molecules in triatomine midguts, one of the most important tissues in triatomines due to its continual contact with a variety of microorganisms, such as *T. cruzi* and microbiota [6].

The triatomine microbiota is involved in vectorial competence via direct contact with *T. cruzi* or by competing for resources in the gut [7]. The microbiota can also interfere indirectly in pathogen development by increasing the expression of antiparasitic molecules and humoral immune defense factors [8,9]. Because vectorial competence depends on a suitable balance between *T. cruzi* and the microbiota [10], the parasite appears to manipulate vector midgut immune responses to modulate the microbiota population in triatomines [11]. In insects, humoral defenses consist of several immune factors, including antimicrobial peptides (AMPs) [12]. Therefore, investigating the AMPs present in the digestive tracts of triatomines may aid in understanding the role these molecules play in the interactions between insect vectors and microorganisms.

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Some AMPs from triatomines have been characterized [13,14], and the production of these molecules is important for maintaining microbiota homeostasis in midguts [15]. However, the role that antibacterial molecules play in interactions between *T. cruzi* and bacteria found in anterior midguts in triatomines (such as *T. infestans*) still remains unknown.

A sequence encoding an unknown protein in the *T. infestans* intestinal transcriptome was found to be upregulated upon *T. cruzi* infection [16]. We characterized the protein encoded by this transcript (TiAP – *T. infestans* antimicrobial protein) and found the protein to be a new molecule with antimicrobial properties. In this work, we demonstrate that TiAP interferes with the bacterial load in *T. infestans* anterior midguts, facilitating the establishment of *T. cruzi* in the intestinal tracts of *T. infestans*.

2. Materials and methods

2.1. Materials

Bacteria and vector: *Escherichia coli* DH5α (F, endA1, hsdR17, sup E44, thi1, k, recA1, gyrA96, Ø 80 d lacZD15) was used as the host for recombinant DNA manipulation and was purchased from Invitrogen (Carlsbad, CA). *E. coli* C43 (F – *ompT hsdSB (rB- mB-) gal dcm* (DE3) harboring pLysS (CmR)) was kindly provided by Dr. Itabajara da Silva Vaz Júnior from the Federal University of Rio Grande do Sul. Primers were purchased from Exxtend (São Paulo, Brazil). The pET-14b expression vector was purchased from Novagen (Madison, WI). Modification enzymes: Restriction enzymes Nde I and BamHI were purchased from Promega (Madison, WI) and Fermentas (Hanover, MD), respectively. Taq DNA polymerase was purchased from Fermentas (Hanover, MD). Chromatography columns: HiTrap SP FF and Sephadex G-75 columns were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Thrombin (EC 3.4.21.5) was purchased from Sigma (St. Louis, MO, USA).

2.2. Methods

2.2.1. Ethics statement

Experimental protocols for mice infections were carried out in accordance with the guidelines of the Ethics Committee in Animal Research from the Federal University of São Paulo (CEP – UNIFESP), approved under registry 5358-2014 and guidelines from the Ethics Committee in Animal experimentation from Federal University of Minas Gerais (CETEA/UFMG), approved under registration number 115/2011.

2.2.2. Bioinformatics analysis

The TiAP amino acid sequence was submitted to the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.html) created by a structural biology group at the Nebraska University Medical Center [17]. This database uses an algorithm to determine the probability that a peptide or a protein is an antimicrobial molecule.

2.2.3. Double-stranded RNA synthesis and release

The TiAP DNA fragment was amplified by PCR using TiAP primers conjugated with the T7 promoter region (TiAPfwd: 5' – GGGAATTCCATATAGTATCCAAAAACTGCATGC - 3'; TiAPrev: 5′-AACATTTATGGAAGATAAGGATCCGCG -3′). This procedure was also used for the mouse epithelium keratin gene (NM_027574) MKfwd: 5′-GGGGTCTCCTCTCTGGAAAC-3′ and MKrev: 5′-ATTAG-CAGCCGTGGAAGAGA-3'. PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN) and used as templates for double-stranded RNA (dsRNA) synthesis using the T7 Ribomax™ Express RNAi system. After synthesis, dsRNAs were digested, isopropanol-precipitated and suspended in sterile 0.9% NaCl at a concentration of 5 μg/μL. Concentrations of dsRNA solutions were calculated by measuring absorbance at 260 nm in a NanoVue spectrophotometer (GE Healthcare).

Using a microinjector (Nanoinjector, Drummond, USA), aliquots of dsRNA (5 μg) were injected twice into the thoracic hemolymphs of *T. infestans* at the third nymphal stage (7 days of starvation after molt) [18]. Control groups included insects injected with 0.9% NaCl and insects injected with an equal amount of mouse epithelium keratin dsRNA (dsMK). This procedure was repeated forty-eight hours after the first injection. Forty-eight hours after the second dsRNA delivery, insect anterior midguts (including the cardia) were dissected, and total RNA was extracted from these tissues using TRIzol (Invitrogen) and quantified using a NanoVue spectrophotometer (GE Healthcare). Then, 1 μg of RNA was treated with 1 unit of DNase (Fermentas) for 1 h at 37 °C. Reactions were stopped by adding EDTA and heating for 10 min at 65 °C. cDNA synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's guidelines. Finally, PCR reactions were performed, and amplicons were loaded onto a 1% agarose gel for verification of TiAP transcript silencing.

2.2.4. T. infestans infection

T. infestans were infected with bloodstream trypomastigote forms from a *T. cruzi* CL strain. First, the parasites (obtained from LLC-MK2 cells) were used to infect B6.129S7-IFNg KO mice. Then, third instar nymphs were fed on anesthetized (ketamine 150 mg/kg and xylazine 10 mg/kg) *T. cruzi* - infected mice (1.6×10^6 parasites/mL). Each nymph ingested approximately 5 μL blood.

2.2.5. T. cruzi load determination in knocked down T. infestans

Eight days after dsRNAs delivery, *T. infestans* were infected with bloodstream trypomastigote forms from a *T. cruzi* CL strain. Seven nymphs from each group were infected as described above. Insects were dissected three hours after infection, and the number of parasites in the anterior midgut of each insect was determined by light microscopy (Olympus) according to the method described by Brener [19].

2.2.6. qPCR for determination of bacterial load in anterior midgut from T. infestans

qPCR was performed using cDNA pooled from four anterior midguts (insects injected with NaCl, dsMK and dsTiAP followed by *T. cruzi* infection and dissection as described above). Three biological replicates were tested for each treatment. In addition, a universal primer set that targets a region of 16S rDNA (16SrDNAfwd: 5′- AGAGTTTGATCCTGGCTCAG-3'; 16SrRNArev: 5′-CATGCTGCCTCCCGTAGGAGT-3′) conserved in several bacteria [20] was used to determine the bacterial load within *T. infestans* anterior midguts. *T. infestans* 18S ribosomal RNA primers were used as the internal control.

Experiments were performed using SYBR® Green PCR Master Mix (Applied Biosystems) in a StepOnePlus PCR system (Applied Biosystems). The qPCR reaction consisted of 1.2 μL of 10-fold diluted cDNA (5 ng), 12.5 μL of SYBR® Green and 0.2 μM (final concentration) of each primer. The PCR program consisted 40 cycles at 94 °C (15 s) and 60 °C (1 min) and was followed by a melt curve generation step. Melt curves were analyzed to check amplification specificity. The calibrator was the NaCl injected group. qPCR analysis was performed according to the method described by Livak and Schmittgen [21], using delta delta Ct calculations to determine relative quantities of transcripts. Reactions were performed in duplicate (for each biological sample), and all values are represented as the means ± standard errors. One-Way ANOVA followed by a Tukey multiple comparison test was used to analyze the

experiment. Significant differences were accepted at $P < 0.05$.

2.2.7. Cloning of the TiAP DNA fragment into the pET-14b vector

The DNA fragment encoding TiAP was amplified by PCR using a cDNA preparation from a *T. infestans* midgut as the template. The 50 μL reaction volume contained 5 pmol of gene-specific primers, 200 μM DNTPs, 1.5 mM $MgCl_2$, and 5 U Taq DNA polymerase (Fermentas). PCR conditions were: 94 °C for 5 min, 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s, repeated a total of 35 cycles. Final extension of the DNA was performed at 72 °C for 10 min. The primers used were: TiAP forward: 5′-GCCAATTTCCA-TATGTATCCAAAAACTGCATGCG-3′ and TiAP reverse: 5′-CGCGCATCCTGTTATCTTCCATAAATGTTGAT-3'. The PCR product was purified using a QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's guidelines. The purified DNA fragment was digested with NdeI and BamHI restriction enzymes and ligated into a similarly digested pET-14b vector (Invitrogen). Midipreps were performed according to the method described by Sambrook [22].

2.2.8. Expression of recombinant TiAP

TiAP was expressed in *E. coli* C43 as a protein fused to an N-terminal 6 X His tag. Previously, recombinant TiAP was produced in 1 L LB broth medium containing 200 μg/mL ampicillin, 34 μg/mL chloramphenicol and one isolated colony of transformed *E. coli* C43. rTiAP expression was induced with 1 mM IPTG (at OD = 0.6) for 7 h at 30 °C. Cells were harvested by centrifugation (3500× g, 20 min, 4 °C) and suspended in 100 mL of 50 mM sodium phosphate buffer, pH 7.0. Cells were lysed using a French press (1500 psi), and the culture supernatant was clarified via centrifugation (13,000× g, 45 min, 4 °C).

2.2.9. Purification and processing of recombinant TiAP

Recombinant TiAP in the culture supernatant was purified by loading the supernatant onto a HiTrap SP column, which was connected to an AKTA™ purifier system (GE Healthcare). The column was previously equilibrated with 50 mM sodium phosphate buffer, pH 7.0. The protein was eluted with a linear NaCl gradient (0–1 M) in 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl at a flow rate of 1.5 mL/min. The eluted protein solution was incubated with 1 unit of thrombin (Sigma-Aldrich) for 24 h at 4 °C for His tag removal. After digestion, rTiAP was concentrated in a 3 kDa NMWL Amicon Ultracel membrane, then buffer exchanged with 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl. The rTiAP was further purified on a Sephadex G-75 gel filtration column. The elution profile was monitored by measuring absorbance at 280 nm, and the purification steps were analyzed by SDS–PAGE electrophoresis [23].

2.2.10. Trypanocidal assay

Bloodstream trypomastigotes from the CL strain of *T. cruzi* were obtained from heart punctures of infected B6.129S7-IFNg KO mice [24]. To analyze the effect of TiAP *T. cruzi*, 1.7×10^7 parasites/mL were incubated for 3 h at 25 °C with different concentrations of TiAP (0.071–1.14 μM). After incubation with TiAP, trypomastigote death rates were determined by using light microscopy to directly quantify the number of live and viable parasites (parasites displaying typical motility) [25].

2.2.11. Bacteriostatic assay

Bacteriostatic assays were performed according to the method described by Fogaça et al. [26]. *E. coli* BL21 (DE3) containing pLysS and *Micrococcus luteus* were incubated at $OD_{550} = 0.001$ with different concentrations of TiAP (0.12–8 μM) on a 96-well microplate. The plate was incubated at 37 °C, 300 rpm until the cultures reached an OD_{500} of 0.3 (determined with a microplate reader). Control groups without protein were used.

3. Results

A transcript encoding a putative unknown protein was found to be upregulated in the anterior midgut transcriptome from *T. cruzi*-infected *T. infestans* [16]. The nucleotide sequence of the mature protein created from this transcript (without a signal peptide) was 327-bp ORF and encoded a secreted protein with a calculated molecular weight of 12.5 kDa and an isoelectric point (pI) of 9.74. Approximately 20% of the protein's amino acids were positively charged (Fig. 1), suggesting that this protein could possess antimicrobial properties [27]. According to prediction analysis with an antimicrobial peptide database (http://aps.unmc.edu/AP/main.html), this sequence encodes an antimicrobial protein named TiAP - "*T. infestans* antimicrobial protein".

Because TiAP is upregulated in *T. infestans* upon infection by *T. cruzi* [16], we knocked down this transcript to determine its function following infection. The transcript was efficiently silenced after injection of dsTiAP into *T. infestans* 3rd instars nymphs; no silencing was observed in control groups (Fig. 2A). After TiAP knockdown, nymphs were infected with bloodstream trypomastigotes, and the quantity of *T. cruzi* in anterior midguts was determined 3 h after infection. Surprisingly, the number of parasites was lower in silenced insects compared to control groups (Fig. 2B), suggesting that TiAP is important for the establishment of *T. cruzi* in *T. infestans* anterior midguts.

In this regard, TiAP might target other microorganisms present in the *T. infestans* anterior midgut, such as some bacterial species present in the tissue. Therefore, qPCR was performed to determine the bacterial load in *T. infestans* anterior midguts using cDNAs from TiAP-silenced and unsilenced insects 3 h after *T. cruzi* infection (Fig. 3). The quantity of bacteria in anterior midguts was significantly higher in silenced insects (approximately 600-fold) compared to the control groups (*T. infestans* injected with NaCl or dsMK).

The cDNA fragment encoding TiAP was cloned and expressed to determine the role that the protein plays in interactions with microorganisms. First, expression of the cloned fragment was attempted in *E. coli* BL21 (DE3) pLysS; however, no protein expression was detected in this strain. Recombinant TiAP (rTiAP) was successfully produced in *E. coli* C43 and purified by ion exchange chromatography (Fig. 4A). SDS–PAGE analysis revealed a major band of His tag – rTiAP, which migrated at 14.1 kDa (Fig. 4C, lane 1), as well as a slight contaminant protein. TiAP was digested with thrombin to remove the His tag, and the digested protein was purified by gel filtration (Fig. 4B). The resulting sample showed a major band at 12.5 kDa (rTiAP lacking His tag) in SDS–PAGE electrophoresis (Fig. 4C, lane 2).

To confirm the role that TiAP plays in interactions with microorganisms, purified rTiAP was incubated with bloodstream *T. cruzi* trypomastigotes. Several different concentrations of rTiAP (0.12–8 μM) had no effect on parasite survival (Fig. 5).

Bacterial growth inhibition experiments were also performed by incubating various concentrations of rTiAP (0.12–8 μM) with *E. coli*

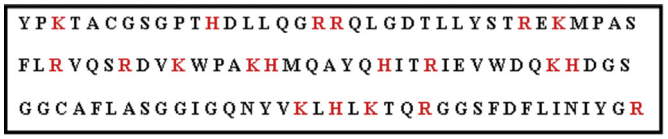

Fig. 1. Analysis of the predicted amino acid sequence from mature TiAP (lacking signal peptides). Positively charged residues are in red.

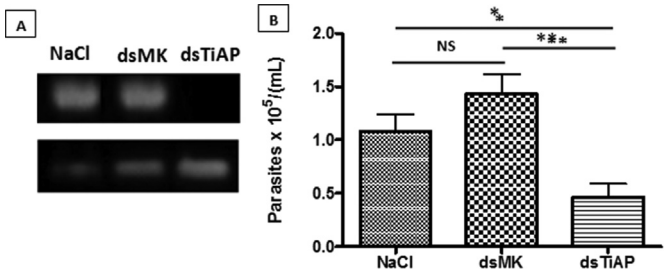

Fig. 2. Knockdown of TiAP transcript by interference RNA and determination of *T. cruzi* load in *R. prolixus* anterior midguts. (A) Agarose gel 1% (p/v) from amplicons, using cDNAs from anterior midguts of insects injected with saline solution (NaCl), dsMK (an unrelated gene – mouse epithelium keratin) or dsTiAP- (TiAP gene) as templates. PCRs were performed using primers specific to TiAP and 18S ribosomal RNA (endogenous control). (B) *T. cruzi* amounts in *T. infestans* anterior midguts. Infections were performed eight days after TiAP silencing, and parasites were counted 3 h after infection by feeding on infected mice (1.6×10^6 parasites/mL). One – Way ANOVA followed by Tukey's multiple comparison test was performed for statistical analysis; differences were considered significant at $P < 0.05$. Asterisks represent significant differences (*$P < 0.05$; **$P < 0.01$). NS indicates a non-significant difference.

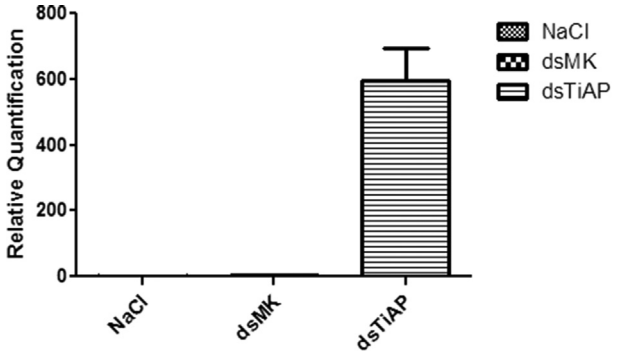

Fig. 3. qPCR for determination of bacterial load in *T. infestans* anterior midguts after TiAP transcript knockdown. cDNAs from insects injected with saline solution (NaCl), injected with dsMK (non-related gene – mouse epithelium keratin) and TiAP silenced insects (dsTiAP) were used for analysis (three biological samples were used for each group). All data were normalized to 18S ribosomal RNA, representing the mean ($n = 3$) of identical triplicates ± standard error. One – Way ANOVA followed by Tukey's multiple comparison test was performed for statistical analysis, and differences were considered significant at $P < 0.05$. Asterisks indicate significant differences (***$P < 0.001$). NS indicates a non-significant difference.

or *M. luteus*. Although the protein was not inhibitory to *M. luteus*, it was able to interfere with *E. coli* growth at a Minimum Inhibitory Concentration (MIC) of 1.2 μM (see Table 1).

4. Discussion

According to our previous results, it is interesting to highlight that the TiAP transcript did not match known proteins in several databases, suggesting that TiAP represents a new protein discovered in the *T. infestans* anterior midgut [16]. Thus, elucidation of the role of this putative protein merited further investigation, as the expression of this protein is highly modulated by *T. cruzi* infection. Furthermore, the protein appears to be involved in vector - microorganisms relationships.

The present study describes the characterization of TiAP in the anterior midgut of the Chagas disease vector *T. infestans*. The cationic characteristics of the translated protein suggests that it could be an antimicrobial protein, as cationic characteristics have been described for some antimicrobial proteins in the literature [27]. The amino acid sequence of the protein was therefore analyzed in an antimicrobial peptide database, which predicted the protein to be a novel antimicrobial protein. In fact, some cationic antimicrobial peptides have been described for triatomine insects [13,14,28]. Because TiAP is upregulated in *T. cruzi* - infected *T. infestans*, we knocked down TiAP transcript levels to determine the role of the protein after infection. Interestingly, the number of *T. cruzi* in silenced insects was lower compared to the control groups (Fig. 2B), suggesting that TiAP is critical for the establishment of *T. cruzi* in *T. infestans* anterior midguts. TiAP could therefore target the microbiota in *T. infestans* to maintain homeostasis of anterior midgut, as these organisms closely interact with *T. cruzi* [29,30]. In fact, the parasite modulates molecules in the anterior midguts of other triatomines as well: *T. cruzi* modulates a Kazal-type inhibitor in *Rhodnius prolixus*, decrease the microbiota load in this compartment [10]. To study the effect of TiAP on the microbiota, qPCR was performed to determine the amount of bacteria in *T. infestans* anterior midguts using cDNAs from TiAP-silenced and unsilenced insects 3 h post *T. cruzi* infection (Fig. 3). The amount of bacteria in anterior midguts was significantly higher in silenced insects (approximately 600-fold) compared to the control groups, indicating that TiAP can help to regulate microbiota populations in anterior midguts. Furthermore, these results suggest that TiAP is important to *T. cruzi* in the first few hours after infecting *T. infestans*.

Microbiota can compete with *T. cruzi* for nutrients [7], or these bacteria can produce trypanolytic factors [31]. Thus, the population of microbiota in the anterior midgut could determine the environment where the parasites must grow [32]. Therefore, to facilitate its development inside *T. infestans*, *T. cruzi* must maintain an equilibrium with existing anterior midgut bacteria [33].

T. cruzi – bacteria interactions seem to be time-specific, as triatomine microbiota are kept at low levels in the first few hours following infection [34,35]. In some cases, slow development can be caused by the long generation time of bacteria, such as *Rhodococcus triatomae* [36]. In addition, the intestinal environment of the insect can be modulated by *T. cruzi*, controlling the microbiota for its own benefit. *T. cruzi* is able to increase the antibacterial activity in another triatomine insect (*R. prolixus*) to decrease midgut microbiota; this ability is crucial for establishment of the parasite [11]. Other protozoans, such as *Trypanosoma rangeli*, can also enhance antibacterial activity in triatomines, targeting bacterial species that are harmful to the parasite [37].

The antibacterial activity in triatomines consists of antimicrobial peptides, such as lysozyme, defensin and prolixicin [13,14,28]. We believe that TiAP belongs to this group of molecules, modulating the microbiota to allow the establishment of *T. cruzi* establishment in the *T. infestans* anterior midgut in the first few hours following infection.

To assess the effect of TiAP on *T cruzi* bloodstream trypomastigotes *in vitro*, we expressed and purified rTiAP. Trypomastigotes are the infective stage of the parasite ingested with blood [38]. No trypanocidal effect was observed with the concentrations of rTiAP tested (Fig. 5), indicating that this protein targets other microorganisms in *T. infestans* anterior midguts. It is important to note that rTiAP could not be expressed in *E. coli* BL21 (DE3) pLysS but was efficiently produced in *E. coli* C43, a BL21 (DE3) that contains genetic mutations which provide tolerance to toxic proteins and prevent cell death [39]. Together with our previous results, this observation suggested that TiAP may target bacteria.

To determine the interactions between TiAP and bacteria, we performed growth inhibition assays to determine the MIC of the protein for *E. coli* and *M. luteus*, both well-known bacteria commonly used in antimicrobial experiments [40]. It is interesting that TiAP was only able to inhibit the growth of *E. coli* (MIC = 1.2 μM), a Gram-negative bacterium. Indeed, the diversity of microbiota is large within *Triatoma* genus and includes some

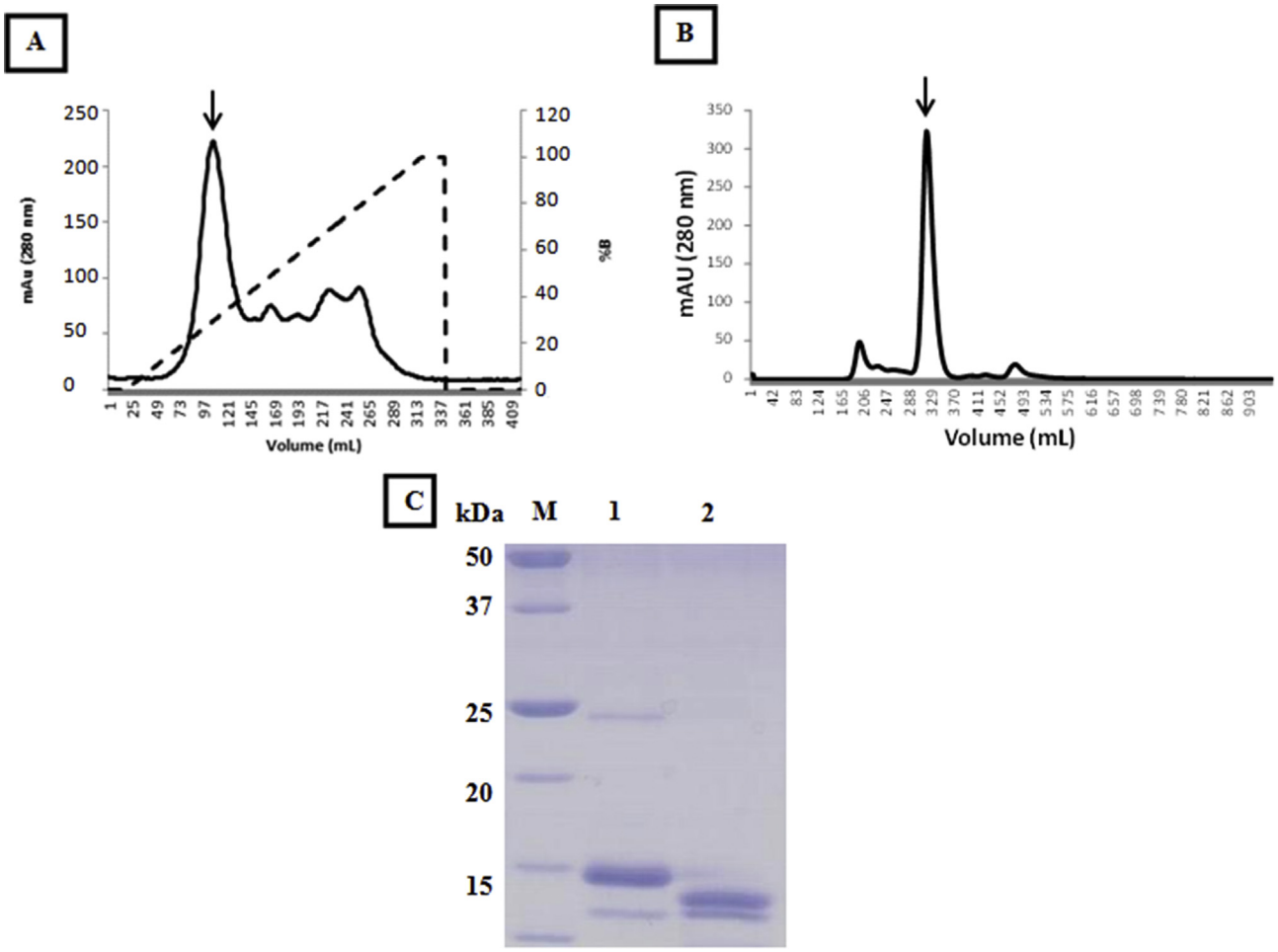

Fig. 4. rTiAP purification. The protein was applied to a HiTrap SP column (A), and fractions containing TiAP (indicated by a black arrow) were pooled, digested with thrombin, concentrated and loaded onto a Sephadex G-75 gel filtration column (B). (C) rTiAP analysis by SDS–PAGE (15%). M – molecular weight marker (PPPUS), (1) His-TiAP pool from gel filtration chromatography (indicated by a black arrow in Figure 4B); (2) thrombin processed rTiAP (lacking a His-tag).

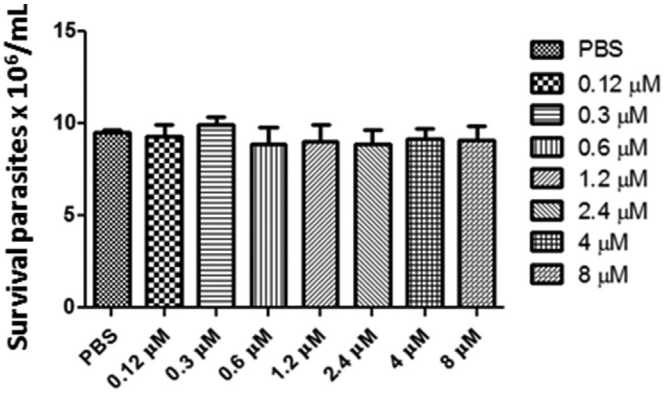

Fig. 5. Effect of TiAP on bloodstream trypomastigotes of *Trypanosoma cruzi*. The parasites were evaluated for viability 3 h after incubation with different concentrations of TiAP (0.12–8 µM) at 25 °C.

Table 1
Minimum inhibitory concentrations of TiAP for *Escherichia coli* and *Micrococcus luteus*.

Bacteria (Gram)	MIC (µM)
Escherichia coli (–)	1.2
Micrococcus luteus (+)	–

extracellular Gram-negative bacteria species [8,29]. Thus, TiAP (a secreted protein) could be involved in modulating these bacteria, which interfere with *T. cruzi* survival or proliferation within *T. infestans* anterior midgut lumens.

5. Conclusions

In summary, the present work describes the characterization of a new protein that presents antimicrobial properties in the anterior midgut of *T. infestans*. Based on our results, we propose that TiAP regulates the *T. infestans* microbiota, favoring the establishment of *T. cruzi* in anterior midguts in the first few hours following infection. Moreover, our data provide insight into the modulation of an intestinal molecule by *T. cruzi*, showing new perspectives of aspects of vector-parasite relationships.

Authors' contributions

AST DSB RNA MHP and AAG planned the experiments. DSB CMG and RCF performed the experiments. DSB and AST analyzed the data. AST MHP RNA and AAG contributed reagents and other essential materials. DSB and AST wrote the manuscript. All of the authors approved the final manuscript.

Conflict of interest

The authors declare there is no conflict of interest.

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References

[1] M.L. Avila, V. Tekiel, G. Moretti, S. Nicosia, J. Bua, M.L. Lammel, M.M. Stroppa, N.M.G. Burgos, D.O. Sanchez, Gene Discovery in *Triatoma infestans*, Parasites Vectors 4 (2011), http://dx.doi.org/10.1186/1756-3305-4-39.

[2] C.R.J. Chagas, Nova tripanosomíase humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen. n. esp., agente da nova entidade mórbida do homem, Mem. Inst. Oswaldo Cruz 1 (1909) 159–218.

[3] M. Gumiel, F.F. Mota, V.S. Rizzo, O. Sarquis, D.P. Castro, M.M. Lima, E.S. Garcia, N. Carels, P. Azambuja, Characterization of the microbiota in the guts of *Triatoma brasiliensis* and *Triatoma pseudomaculata* infected by *Trypanosoma cruzi* in natural conditions using culture independent methods, Parasites Vectors 8 (2015), http://dx.doi.org/10.1186/s13071-015-0836-z.

[4] R.J. Ursic-Bedoya, C.A. Lowenberger, *Rhodnius prolixus*: Identification of immune-related genes up-regulated in response to pathogens and parasites using suppressive subtractive hybridization, Dev. Comp. Immunol. 31 (2007) 109–120.

[5] M.M.A. Whitten, F. Sun, I. Tew, G.A. Schaub, C. Soukou, A. Nappi, N. Ratcliffe, Differential modulation of *Rhodnius prolixus* nitric oxide activities following challenge with *Trypanosoma rangeli*, *Trypanosoma cruzi* and bacterial cell wall components, Insect Biochem. Mol. Biol. 37 (2007) 440–452.

[6] E.S. Garcia, F.A. Genta, P. Azambuja, G.A. Schaub, Interactions between intestinal compounds of triatomines and *Trypanosoma cruzi*, Trends Parasitol. 26 (2010) 499–505.

[7] E.S. Garcia, D.P. Castro, M.B. Figueiredo, P. Azambuja, Immune homeostasis to microorganisms in the guts of triatomines (Reduviidae)–a review, Mem. Inst. Oswaldo Cruz 105 (2010) 605–610.

[8] P. Azambuja, E.S. Garcia, N.A. Ratcliffe, Gut microbiota and parasite transmission by insect vectors, Trends Parasitol. 21 (2005) 568–572.

[9] B. Weiss, S. Aksoy, Microbiome influences on insect host vector competence, Trends Parasitol. 27 (2011) 514–522.

[10] T.S. Soares, D.S. Buarque, B.R. Queiroz, C.M. Gomes, G.R.C. Braz, R.N. Araújo, M.H. Pereira, A.A. Guarneri, A.S. Tanaka, A Kazal-type inhibitor is modulated by *Trypanosoma cruzi* to control microbiota inside the anterior midgut of *Rhodnius prolixus*, Biochimie 112 (2015) 41–48.

[11] D.P. Castro, C.S. Moraes, M.S. Gonzalez, N.A. Ratcliffe, P. Azambuja, E.S. Garcia, *Trypanosoma cruzi* immune response modulation decreases microbiota in *Rhodnius prolixus* gut and is crucial for parasite survival and development, PLoS ONE 75 (2012), http://dx.doi.org/10.1371/journal.pone.0036591.

[12] E.S. Garcia, D.P. Castro, M.B. Figueiredo, F.A. Genta, P. Azambuja, *Trypanosoma rangeli*: a new perspective for studying the modulation of immune reactions of *Rhodnius prolixus*, Parasites Vectors 2 (2009), http://dx.doi.org/10.1186/1756-3305-2-33.

[13] L. Lopez, G. Morales, R. Ursic, M. Wolff, C.A. Lowenberger, Isolation and characterization of a novel insect defensin from *Rhodnius prolixus*, a vector of Chagas disease, Insect. Biochem. Mol. Biol. 33 (2003) 439–447.

[14] R. Ursic-Bedoya, J. Buchhop, J.B. Joy, R. Durvasula, C. Lowenberger, Prolixicin: a novel antimicrobial peptide isolated from *Rhodnius prolixus* with differential activity against bacteria and *Trypanosoma cruzi*, Insect. Mol. Biol. 20 (2011) 775–786.

[15] C.S. Vieira, P.J. Waniek, D.P. Mattos, D.P. Castro, C.B. Mello, N.A. Ratcliffe, E.S. Garcia, P. Azambuja, Humoral responses in *Rhodnius prolixus*: bacterial feeding induces differential patterns of antibacterial activity and enhances mRNA levels of antimicrobial peptides in the midgut, Parasites Vectors 7 (2014), http://dx.doi.org/10.1186/1756-3305-7-232.

[16] D.S. Buarque, G.R.C. Braz, R.M. Martins, A.M. Tanaka-Azevedo, C.M. Gomes, F.A.A. Oliveira, S. Schenkman, A.S. Tanaka, Differential expression profiles in the midgut of *Triatoma infestans* infected with *Trypanosoma cruzi*, PloS One 8 (2013), http://dx.doi.org/10.1371/journal.pone.0061203.

[17] Z. Wang, G. Wang, APD: the antimicrobial peptide database, Nucleic Acids Res. 32 (2004) D590–D592.

[18] R.M.M. Paim, R.N. Araújo, M.J. Lehane, N.F. Gontijo, M.H. Pereira, Application of RNA interference in triatomine (Hemiptera: Reduviidae) studies, Insect Sci. 20 (2013) 40–52.

[19] Z. Brener, Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*, Rev. Inst. Med. Trop. São Paulo 4 (1962) 389–396.

[20] M.A. Nadkarni, F.E. Martin, N.A. Jacques, N. Hunter, Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set, Microbiology 148 (2002) 257–266.

[21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and the 2-[Delta][Delta]CT method, Methods 25 (2001) 402–408.

[22] J. Sambrook, in: F. Fritsch, T. Maniatis (Eds.), Molecular Cloning: a Laboratory Manual, second ed., 1989.

[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[24] M.N. Meirelles, T.C. Araujo-Jorge, W. de Souza, Interaction of *Trypanosoma cruzi* with macrophages *in vitro*: dissociation of the attachment and internalization phases by low temperature and cytochalasin B, Z. Parasitenkd. 68 (1982) 7–14.

[25] C.F. Silva, M.B. Meuser, E.M. de Souza, M.N.L. Meirelles, C.E. Stephens, P. Som, D.W. Boykin, M.N.C. Soeiro, Cellular effects of reversed amidines on *Trypanosoma cruzi*, Antimicrob. Agents Chemother. 51 (2007) 3803–3809.

[26] A.C. Fogaça, D.M. Lorenzini, L.M. Kaku, E. Esteves, P. Bulet, S. Daffre, Cysteine-rich antimicrobial peptides of the cattle tick *Boophilus microplus*: isolation, structural characterization and tissue expression profile, Dev. Comp. Immunol. 28 (2004) 191–200.

[27] T.C. Assumpção, I.B.M. Francischetti, J.F. Andersen, A. Schwarz, J.M. Santana, J.M.C. Ribeiro, An insight into the sialome of the blood-sucking bug *Triatoma infestans*, a vector of Chagas' disease, Insect Biochem. Mol. Biol. 38 (2008) 213–232.

[28] C.A. Araújo, P.J. Waniek, P. Stock, C. Mayer, A.M. Jansen, G.A. Schaub, Sequence characterization and expression patterns of defensin and lysozyme encoding genes from the gut of the reduviid bug *Triatoma brasiliensis*, Insect Biochem. Mol. Biol. 36 (2006) 547–560.

[29] F.F. Mota, L.P. Marinho, C.J.C. Moreira, M.M. Lima, C.B. Melo, E.S. Garcia, N. Carels, P. Azambuja, Cultivation-independent methods reveal differences among bacterial gut microbiota in triatomine vectors of Chagas disease, PLoS Negl. Trop. Dis. 6 (2012), http://dx.doi.org/10.1371/journal.pntd.0001631.

[30] D.P. Castro, C.S. Moraes, M.S. Gonzalez, I.M. Ribeiro, T.C. Tomassini, P. Azambuja, E.S. Garcia, Physalin B inhibits *Trypanosoma cruzi* infection in the gut of *Rhodnius prolixus* by affecting the immune system and microbiota, J. Insect Physiol. 58 (2012) 1620–1625.

[31] P. Azambuja, D. Feder, E.S. Garcia, Isolation of *Serratia marcescens* in the midgut of *Rhodnius prolixus*: impact on the establishment of the parasite *Trypanosoma cruzi* in the vector, Exp. Parasitol. 107 (2004) 89–96.

[32] G.A. Vallejo, F. Guhl, G.A. Schaub, Triatominae – *Trypanosoma cruzi*/*T. rangeli*: Vector–parasite interactions, Acta Trop. 110 (2009) 137–147.

[33] C. Genes, E. Baquero, F. Echeverri, J.D. Maya, O. Triana, Mitochondrial dysfunction in *Trypanosoma cruzi*: the role of *Serratia marcescens* prodigiosin in the alternative treatment of Chagas disease, Parasites Vectors 4 (2011), http://dx.doi.org/10.1186/1756-3305-4-66.

[34] S. Eichler, G.A. Schaub, Development of symbionts in triatomine bugs and the effects of infections with trypanosomatids, Exp. Parasitol. 100 (2002) 17–27.

[35] B.S. McGwire, M.M. Kulkarni, Interactions of antimicrobial peptides with Leishmania and trypanosomes and their functional role in host parasitism, Exp. Parasitol. 126 (2010) 397–405.

[36] A.F. Yassin, *Rhodococcus triatomae* sp. nov., isolated from a blood-sucking bug, Int. J. Syst. Evol. Microbiol. 55 (2005) 1575–1579.

[37] C.L. Vieira, D.P. Matos, P.J. Waniek, J.M. Santangelo, M.B. Figueiredo, M. Gumiel, F.F. Mota, D.P. Castro, E.S. Garcia, P. Azambuja, *Rhodnius prolixus* interaction with *Trypanosoma rangeli*: modulation of the immune system and microbiota population, Parasites Vectors 8 (2015), http://dx.doi.org/10.1186/s13071-015-0736-2.

[38] F.M. Lima, P. Oliveira, R.A. Mortara, J.F. Silveira, D. Bahia, The challenge of Chagas' disease: has the human pathogen *Trypanosoma cruzi* learned to modulate signaling events to subvert host cells? New Biotech. 27 (2010) 837–843.

[39] L. Dumont-Seignovert, G. Cariot, L. Vuillard, The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3), Protein Expr. Purif. 37 (2004) 203–206.

[40] P. Bulet, J.L. Dimarcq, C. Hetru, M. Lagueux, M. Charlet, G. Hegy, A. van Dorsselaer, J.A. Hoffmann, A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution, J. Biol. Chem. 268 (1993) 14893–14897.

Web reference

[41] http://aps.unmc.edu/AP/main.html. Last access: 04/01/2016.