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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