




# Insights into the tracking of the cysteine proteinase B COOH-terminal polypeptide of *Leishmania (Leishmania) amazonensis* by surface plasmon resonance

Raquel Santos-de-Souza<sup>1</sup> · Franklin Souza-Silva<sup>1</sup> · Barbara Cristina de Albuquerque-Melo<sup>1</sup> · Michelle Lopes Ribeiro-Guimarães<sup>1</sup> · Luzia Monteiro de Castro Côrtes<sup>1</sup> · Bernardo Acácio Santini Pereira<sup>1</sup> · Mariana Silva-Almeida<sup>1</sup> · Léa Cysne-Finkelstein<sup>2</sup> · Francisco Odencio Rodrigues de Oliveira Junior<sup>3</sup> · Mirian Claudia de Souza Pereira<sup>3</sup> · Carlos Roberto Alves<sup>1</sup> 

Received: 29 March 2018 / Accepted: 25 January 2019 / Published online: 12 February 2019  
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## Abstract

*Leishmania (Leishmania) amazonensis* has adaptive mechanisms to the host environment that are guided by its proteinases, including cysteine proteinase B (CPB), and primarily its COOH-terminal region (Cyspep). This work aimed to track the fate of Cyspep by surface plasmon resonance (SPR) of promastigotes and amastigotes to gain a greater understanding of the adaptation of this parasite in both hosts. This strategy consisted of antibody immobilization on a COOH1 surface, followed by interaction with parasite proteins and epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64). Pro-CPB and Cyspep were detected using specific polyclonal antibodies against a recombinant Cyspep in both parasite forms. The parasitic supernatants from amastigotes and promastigotes exhibited higher anti-Cyspep recognition compared with that in the subcellular fractions. As the supernatant of the promastigote cultures exhibited resonance unit values indicative of an effective with to E-64, this result was assumed to be Pro-CPB detection. Finally, after using three sequential SPR assay steps, we propose that amastigotes and promastigotes release Cyspep into the extracellular environment, but only promastigotes release this polypeptide as Pro-CPB.

**Keywords** *Leishmania (L.) amazonensis* · Cysteine proteinase · Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane · Surface plasmon resonance

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Raquel Santos-de-Souza and Franklin Souza-Silva contributed equally to this work.

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Section Editor: Sarah Hendrickx

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**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00436-019-06238-5>) contains supplementary material, which is available to authorized users.

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✉ Carlos Roberto Alves  
calves@ioc.fiocruz.br

<sup>1</sup> Laboratório de Biologia Molecular e Doenças Endêmicas, Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Avenida Brasil, 4365, Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil

<sup>2</sup> Laboratório de Imunoparasitologia, Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Avenida Brasil, 4365, Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil

<sup>3</sup> Laboratório de Ultraestrutura Celular, Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Avenida Brasil, 4365, Manguinhos, Rio de Janeiro, RJ 21040-900, Brazil

## Introduction

Leishmaniasis are zoonotic infectious diseases that are caused by more than 20 *Leishmania* species and transmitted by over 90 phlebotomine sand fly species (Family Psychodidae, Subfamily Phlebotominae). These diseases are classified into the following four clinical forms: visceral leishmaniasis, post-kala-azar dermal leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis. The disability-adjusted life years lost due to leishmaniasis approach 2.4 million. There are 1.0 to 1.5 million cases of CL and 500,000 cases of VL each year, and a population of 350 million is at risk. Additionally, leishmaniasis are recognized as important neglected tropical diseases due to their high distribution worldwide and ability to produce deformities, which affect the social and economic status of individuals who suffer from this condition ([http://www.who.int/tdr/publications/disease\\_watch/leish/en/](http://www.who.int/tdr/publications/disease_watch/leish/en/)—accession date: 08/29/2018).

*Leishmania* spp. exhibit a heteroxenous biological cycle, alternating their form between invertebrate and vertebrate hosts, which they infect via vector-borne transmission. These protozoa progress through various morphological stages during their life cycle, including the following two primary forms: (i) the promastigote form, an elongated form with a free flagellum emerging from the anterior region; form is present in the digestive tract of the insect vector; (ii) the amastigote form, a typically ovoid or spherical form with a small flagellum restricted to the flagellar pocket; this form is typically found in macrophages within the phagolysosomal compartment (Teixeira et al. 2013). *Leishmania* spp. parasites experience changes of temperature (26 to 37 °C) and hydrogen potential (pH neutral to pH acid) as they move from one host to another. Previously, these conditions were simulated in vitro, acting as environmental signals that influence the differentiation of promastigotes into amastigotes and the development of virulence factors (Alves et al. 2005; Gomes et al. 2017).

Among these virulence factors, *Leishmania* sp.-derived proteases play a strategic adaptive role in this parasite in mammalian and sand fly hosts (Silva-Almeida et al. 2012). These enzymes have been studied for more than four decades, and due to the discovery of specific inhibitors of these proteases, they are recognized as potential targets for new drugs to treat leishmaniasis (Pereira et al. 2014). The rationale for targeting these proteases is related to their crucial role in host-parasite interactions, participation in immune system modulation, invasion and destruction of host cells and tissues, and aiding in the acquisition of essential nutrients (Klemba and Goldberg 2002) to ensure the survival of the parasite and thus the establishment of infection. Currently, the following four primary classes of proteases have been widely described in *Leishmania* spp.: metalloproteinase, cysteine proteinases, aspartic proteinases, and serine proteinase (Silva-Almeida et al. 2014).

Cysteine proteinases (CPs) are grouped into the subfamilies cathepsins B and L (Sakanari et al. 1997), which are characterized by their hydrolytic mechanism of action based on the thiol nucleophilic group of a cysteine residue in a catalytic triad. Studies in *Leishmania* spp. have demonstrated that CPs are highly expressed in amastigote megasomes (Pral et al. 2003) and on the surface of promastigotes (Alves et al. 2005; Rebello et al. 2009).

Three groups of CPs of *Leishmania* spp. (CPA, CPB, and CPC) have been primarily studied (Silva-Almeida et al. 2014) and have the following specific characteristics: (i) the CPA group comprises a cathepsin L subfamily, which is characterized by a single gene copy and the absence of a long COOH-terminal extension prior to its final processing (Mottram et al. 1992, 1998); (ii) the CPB group also comprises a cathepsin L subfamily, but these cathepsin L proteins contain a long COOH-terminal extension prior to the final processing; these

enzymes are expressed by multiple gene copies, which are arranged in tandem sequences, and their isoforms vary in their substrate specificity and catalytic properties (Brooks et al. 2001); (iii) the CPC group comprises a cathepsins B subfamily and, similar to the CPA group, these enzymes are encoded by a single gene copy and present no COOH-terminal extension (Bart et al. 1995).

The COOH-terminal extension of the CPB group corresponds to a sequence of approximately 100 amino acids, which are released from the CPB through a hydrolytic process that occurs during maturation of the enzyme (Duboise et al. 1994). Once hydrolysed, this polypeptide is secreted into the extracellular environment (Traub-Cseko et al. 1993) and can be observed in the host cell cytoplasm (Alves et al. 2005). CPB has been described as highly immunogenic and may potentially influence the immune response of mice infected with *Leishmania (L.) amazonensis* (Alves et al. 2004; Pereira et al. 2011), *Leishmania (L.) infantum* (Nakhaee et al. 2004), and *Leishmania (L.) donovani* (Hide and Bañuls 2008).

A three-dimensional reconstruction of the COOH-terminal extension, also referred to as Cyspep, was performed, and the final model revealed the existence of certain portions arranged in  $\alpha$ -helix structures and other portions with a linear structure, indicating the possibility of T lymphocyte epitopes contained within the COOH-terminal structure. The activity of T cell epitopes from Cyspep was confirmed by in vitro assays with mononuclear cells from the peripheral blood of patients with leishmaniasis (Alves et al. 2001a) and experimentally in mice infected with *L. (L.) amazonensis* (Alves et al. 2004).

In the present study, we analyzed the fate of Cyspep in amastigotes and promastigotes of *L. (L.) amazonensis* via the association of a specific immune recognition with subcellular fractionation using the surface plasmon resonance (SPR) approach. The data obtained herein provide new evidence for the release of Cyspep by amastigotes and promastigotes into the extracellular environment. Moreover, these data demonstrate that only promastigotes can produce CPB as a proenzyme (pro-CPB).

## Materials and methods

### Chemicals and culture reagents

Detergents (sodium dodecyl sulfate (SDS) and Triton X-100), dithiothreitol (DTT), glycerol, penicillin, streptomycin, Schneider's *Drosophila* medium, protein A-agarose, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), the silver Stain kit Proteo Silver™, nitrocellulose membranes, and cell culture flasks were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The micro-bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical

Co. (Appleton, WI). Fetal calf serum (FCS) was acquired from Gibco, Invitrogen (Brazil). A bacterial expression vector containing the T7lac promoter (pET28a) was purchased from GenScript, Inc. (Piscataway, NJ, USA). Nickel-charged (Ni-NTA) resin was obtained from Qiagen (Brazil). The full-range rainbow™ kit, 12 to 225 kDa, was purchased from GE Healthcare Life's Sciences (Little Chalfont, UK). Coupling agents [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS)] and ethanolamide were purchased from Merck Millipore Corporation (Darmstadt, Alemanha). A carboxylated gold sensor chip (COOH1 chip) was acquired from ICx Nomadics, Inc. (Stillwater, OK). Chemiluminescence was performed using the SuperSignal West Pico Chemiluminescent kit™ Substrate (Thermo Scientific, EUA). All reagents were of analytical grade or higher.

### Parasite cultures

*Leishmania (Leishmania) amazonensis* (MHOM/BR/77/LTB0016) was obtained from the *Leishmania* collection of the Instituto Oswaldo Cruz (CLIOC). Promastigotes were maintained in Schneider's insect medium at pH 7.2 (supplemented with 10% FCS, 200 IU of penicillin, 200-μg/mL streptomycin, and 2% human urine) at 26 °C.

### Axenic amastigote differentiation

In vitro amastigogenesis was performed as previously described (Alves et al. 2005). Approximately  $10^9$  promastigotes/mL (10 mL) in the stationary phase (fourth day) was cultured in Schneider's growth medium, pH 5.5, (supplemented with 20% FCS, 200 IU of penicillin, 200-μg/mL streptomycin) at 32 °C. The differentiation was performed in 25-mL culture flasks and monitored until the complete transformation of the parasite, which occurred within 96 h. The culture supernatants of the transformed amastigotes were collected after centrifugation (3000 ×g, 15 min, 4 °C), followed by filtration through a Millipore membrane (0.22 μM); the filtered supernatants were maintained at -20 °C until use in the SPR assays.

### Subcellular fractionation

Enriched fractions of flagella (Ff) and surface membranes (Mf) were obtained by the subcellular fractionation of either promastigotes or amastigotes, as previously described (De Côrtes et al. 2012).

### Cloning, expression, and purification of Cyspep

A 264-bp fragment corresponding to the COH-terminal region of CPB (rCyspep) was cloned into the pUC57

vector at the site of the EcoRV endonuclease and subcloned into the pET-28a vector (+) between the BamHI-NotI sites. The construct boundaries were as previously described (Pereira et al. 2011): 5'-GGATCCGCACCCAGACCCGT GATGGTGGAGCAGGTGATCTGCTTCGATAA GAACTGCACTCAGGGGTGCAAGAAAACCCT GATCAAGGCGAACGAGTGCCACAAGAACGG GGGAGGAGGCGCCTCTATGATCAAGTGCAG TCCGCAGACGGTGACGATGTGCACGTACTC GAACGAATTCTGCCTGGGCGGGGGGCTGTG CCTCGAGACTCCTGATGGTAAGTGC GCGCC GTACTTTTTGGGCTCGGTCATTAACACCTG TCACTACACGGCCGC-3').

Expression and purification of the rCyspep were performed according to the QIAexpressionist manual (QIAGEN, Valencia, CA). Briefly, *E. coli* BL21-AI™ harboring the recombinant plasmid was grown to an OD<sub>600</sub> of 0.8 in ampicillin-containing Luria-Bertani medium. The expression of rCyspep was then induced by the addition of 1-mM isopropyl beta-D-thiogalactoside, and the cultures were incubated for 3 h at 37 °C. Cells were harvested, resuspended in lysis buffer (100-mM NaH<sub>2</sub>PO<sub>4</sub>, 10-mM Tris-HCl pH 8.0), and disrupted by sonication. The cellular debris was removed by centrifugation (10,000 ×g, 15 min, 4 °C), and rCyspep was purified from the supernatant by the addition of 1 mL of a 50% Ni-NTA slurry (QIAGEN) pre-equilibrated in lysis buffer to 4 mL of the lysate. After incubation (1 h, 4 °C), the lysate-Ni-NTA mixture was loaded into a column. Unbound proteins were removed by washing the column with two column volumes of lysis buffer. Then, the column was washed twice with 4 mL of 100-mM NaH<sub>2</sub>PO<sub>4</sub> and 10-mM Tris-HCl, pH 6.3, and the recombinant protein was eluted with four column volumes of 100-mM NaH<sub>2</sub>PO<sub>4</sub>, pH 4.5. Fractions were analyzed by SDS-PAGE and Western blot. Nitrocellulose membranes were probed with an anti-6xHis tag antibody (New England Biolabs, Ipswich, MA). The purified protein of 14 kDa was stored at -20 °C.

### Mouse anti-rCyspep antiserum

A polyclonal antibody against rCyspep was produced in BALB/c (H-2<sup>d</sup>) mice by successive weekly injections of the recombinant protein (100 μg each). The injections were performed via the dorsal subcutaneous route. The first and second inoculations contained 100 μg of protein in Freund's complete and incomplete adjuvants, respectively, and the third and fourth injections each contained only 100 μg of protein. Blood was collected by cardiac puncture and centrifuged (3000 ×g, 10 min, 4 °C) to obtain hyperimmune serum, and the immunoglobulin G (IgG) fraction was obtained by chromatography on protein A-agarose (Alves et al. 2000). All procedures involving animals were approved by the Animal Ethics Committee of IOC (L052/20151).

## Determination of protein concentrations

The protein concentrations of the extracted samples and rCyspep were determined using the micro-BCA protein assay kit. Bovine serum albumin (BSA) was used as a standard.

## Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis contains sodium dodecyl sulfate (SDS-PAGE) assays under reducing and non-reducing conditions. Proteins samples were initially treated with sample buffer (80-mM Tris-HCl, pH 6.8, 2% SDS (*w/v*), 12% glycerol (*v/v*), 5%  $\beta$ -mercaptoethanol (*v/v*; or not), and 0.05% bromophenol blue (*w/v*)) and boiled for 3 min. Alternatively, assays with polyacrylamide gels (7.5%) had been implemented in non-denaturing conditions (PAGE), without SDS and  $\beta$ -mercaptoethanol in the gel, in the running and sample buffers. After electrophoresis (150 V at 15 mA), the proteins bands were visualized using the silver impregnation method, and the full-range rainbow™ kit was used as a molecular weight marker.

## Western blot analysis

Soluble proteins were resolved by SDS-PAGE and electro-transferred from the gel onto a nitrocellulose membrane (0.2  $\mu$ m). After incubation (4 h, 4 °C) with blocking buffer (PBS, pH 7.2, containing 0.5% Tween 20 and 5% non-fat milk), the membranes were incubated (16 h, 4 °C) with a mouse primary antibody (anti-histidine tail). Subsequently, the membranes were treated with wash buffer (PBS, pH 7.2, containing 0.05% Tween 20) followed by incubation (1 h, 25 °C) with a goat anti-mouse IgG antibody conjugated to peroxidase (1:3000 dilution). After an additional washing step, the immunoreactive bands were visualized by chemiluminescence.

## Surface plasmon resonance assays

SPR assays were performed to quantify the Cyspep produced in the culture supernatant fractions in the membrane and flagellum of amastigotes and promastigotes. The analyses were performed using a COOH1 chip to which 0.5  $\mu$ g of anti-rCyspep IgG was adsorbed, which was purified using the coupling method EDC/NHS. The sensor chip functionalization was performed in eight sequential steps in running buffer (10-mM HEPES, 3-mM EDTA, 150-mM NaCl, 0.005% Tween 20, pH 7.4), and all of the following steps were performed at a continuous flow rate: (1) injection of 10  $\mu$ L of 50-mM HCl at 10  $\mu$ L/min; (2) injection of 100  $\mu$ L of 10-mM CH<sub>3</sub>COOH at 50  $\mu$ L/min; (3) injection of 150  $\mu$ L of the mixture (1:1) 400-mM EDC and NHS at 15  $\mu$ L/min; (4) injection of 100  $\mu$ L of 10-mM CH<sub>3</sub>COOH

at 50  $\mu$ L/min; (5) 100- $\mu$ L injection of mouse IgG at 10  $\mu$ L/min; (6) injection of 100  $\mu$ L of 10-mM CH<sub>3</sub>COOH at 50  $\mu$ L/min; (7) injection of 150  $\mu$ L of 100-mM ethanolamine at 20  $\mu$ L/min; and (8) injection of 150  $\mu$ L of the formulated buffer, which provides maximum stability to the mouse IgG structure at 20  $\mu$ L/min.

Afterward, a calibration curve plotting various concentrations of rCyspep (0.01 to 100  $\mu$ g) was constructed. Next, various concentrations (0.01 to 100  $\mu$ g) of proteins from the cultivation supernatant and either the membrane or flagellum fraction were subjected to interaction with the anti-rCyspep IgG, which was previously immobilized on the chip. BSA at in same concentration range (0.01 to 100  $\mu$ g) was used as the negative control. All analyses were evaluated at a flow rate of 5  $\mu$ L/min. Additional assays were conducted to evaluate the E-64 binding proteins complexed to anti-rCyspep IgG. Various concentrations of E-64 soluble in water (0.001 to 10  $\mu$ M) were injected into a second cycle of interaction after the capture step of rCyspep and/or parasite proteins.

Interaction assays were conducted in the presence of 10-mM phosphate buffer, pH 7.4 (total volume of 100  $\mu$ L with a flow rate of 10  $\mu$ L/min). After each interaction cycle, the chip was regenerated by a new cycle with 0.2-M glycine buffer, pH 3.0 (total volume of 200  $\mu$ L with a flow rate of 50  $\mu$ L/min), followed by a new cycle with phosphate buffer (10-mM phosphate pH 7.4; total of 200  $\mu$ L with a flow rate of 10  $\mu$ L/min). These tests were recorded in a sensorgram and conducted in an optical biosensor transduction SensiQ® Pioneer (Nomadics, Inc., An ICX Company). The initial rate of interaction was determined from the resonance unit (RU) for complex formation. The sensorgrams of association and dissociation for the complex formation were obtained after analysis using the SPR assay Qdat software (ICX Nomadics, USA).

## Data analysis

The quantitative data were presented as the mean  $\pm$  standard deviation. The reliability of the SPR data was confirmed by linear regression using the protein ( $\mu$ g) and E-64 ( $\mu$ M) concentrations for defining the coefficient of determination ( $R^2$ ). The values used to calculate the protein detection on the anti-rCyspep-functionalized chip were obtained by the post-injection RU variation and the RU of dissociation phase (RU<sub>d</sub>). The calibration curve was determined by the ratio of the concentration of rCyspep (abscissa) to RU<sub>d</sub> (ordinate) resulting from the antigen/antibody interaction. This procedure was also used to obtain the values of protein quantity on the chip after the dissociation resonance unit (RU<sub>d</sub>), the amount of protein required to achieve  $\frac{1}{2}$  RU<sub>d</sub>, and the detection constant ( $K_{dec}$ ).



## Results

### Obtaining the recombinant protein and production of the specific IgG

The insertion of the sequence corresponding to the COOH-terminal cysteine-proteinase B region of *L. (L.) amazonensis* into the vector pET28a (+) was performed by GenScript USA, Inc. After the induction of the recombinant protein (rCyspep) and its expression in bacteria transformed with recombinant plasmids (pET28a-rCyspep), the total cell extract was analyzed by immunoblot, revealing the presence of rCyspep in the soluble preparations from selected colonies (Fig. 1). The 14-kDa rCyspep protein was purified by affinity chromatography on a nickel affinity column from this soluble fraction, as indicated by electrophoresis assays: silver impregnation staining, under reducing (Fig. 1) and non-reducing conditions (Fig. 1), and immunodetection with an anti-histidine tail antibody, under reducing conditions (Fig. 1). A single protein band was observed in the native electrophoresis gel assay near the anode (Fig. 1e), as revealed by silver staining.

Subsequently, we also generated hyperimmune sera containing specific polyclonal anti-rCyspep IgG antibodies in BALB/c mice. The immunization schedule used in this study yielded antisera preparations capable of detecting rCyspep via enzyme-linked immunosorbent assay (Supp. 1). The immune and non-immune IgG fractions were purified from the mouse serum preparations by affinity column and subsequently used in the SPR assays.

### Detection of Cyspep in the subcellular fraction and culture supernatants of *L. (L.) amazonensis*

The morphological characterization and evaluation of *cpb* gene expression in various developmental stages of *L. (L.) amazonensis* cultivated in vitro, in both the promastigote and amastigote forms, were analyzed by fluorescence

microscopy (Supp. 2A), flow cytometric analysis (Supp. 2B), and qPCR (Supp. 3). The relative expression of *cpb* in the cDNA samples of completely differentiated amastigotes (96 h) exhibited a higher level of *cpb* transcription in amastigotes (3.4-fold) compared with promastigotes (Supp. 3). Dissociation curves, which were generated to test the specificity of each amplified product, revealed a single melting-temperature peak (Supp. 4).

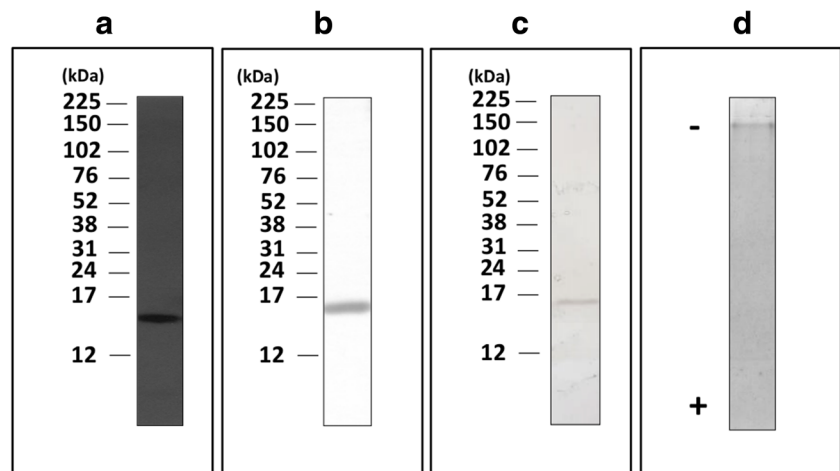
The expression of Cyspep in the membrane and flagella fractions of promastigotes and amastigotes, as well as in the supernatants of the parasites culture, was investigated by the analysis of surface biosensor using a specific IgG against the rCyspep protein as a capture tool of the proteins of the parasites.

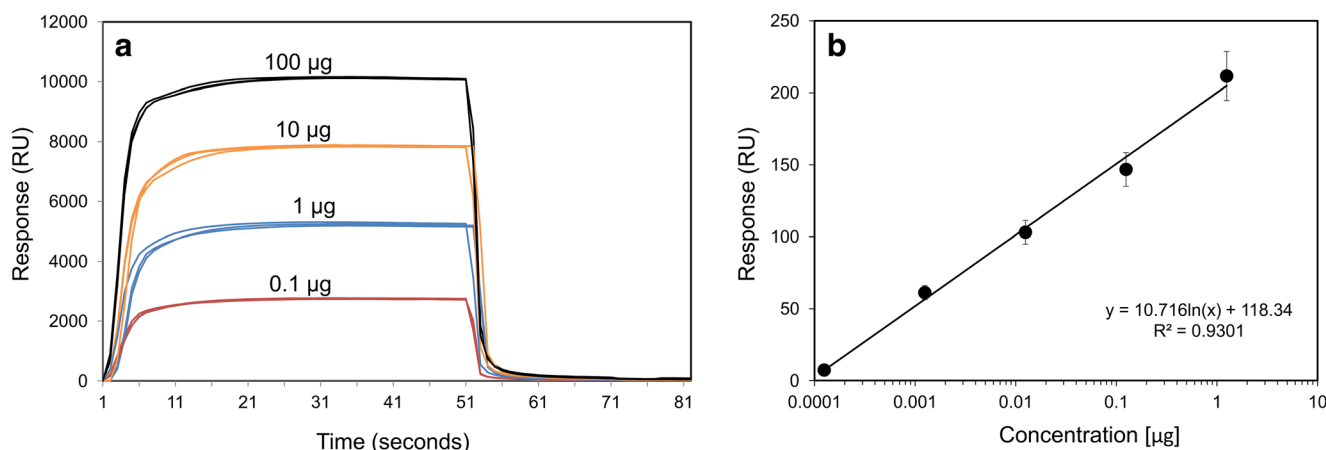
Initially, we standardized the functionalization of the carboxylated sensor chip with a purified anti-rCyspep IgG antibody. The conditions for the immobilization of IgG 0.5- $\mu$ g coupling method using EDC/NHS indicated the presence of  $640 \pm 54$  RU, equivalent to 0.64 ng of protein/ $\text{mm}^2$ , in the chip (Supp. 5). The system was calibrated with purified rCyspep, and a dose/response curve for the quantitative extrapolation of Cyspep proteins present in the parasite fractions was plotted.

Surface biosensor assays demonstrated the binding property of rCyspep to the specific IgG produced during the immunization of the mice. The rCyspep protein was analyzed using a range of concentrations and demonstrated an increase of  $\text{RU}_d$  signal per seconds, indicating the concentration-dependent binding of anti-rCyspep IgG to rCyspep, showing an  $\text{RU}_d$  of 200.06 ( $R^2 = 0.9939$ ). The straight linearization indicated a saturation constant on the chip of approximately  $0.95 \times 10^{-2}$   $\mu$ g (Fig. 2).

In general, the sensorgrams obtained in the antigenicity assays of the parasite subcellular fractions and culture supernatants exhibited  $\text{RU}_d$  values above baseline, which was indicative of the recognition property by the anti-rCyspep IgG antibody in all parasite preparations in a concentration-dependent manner (Figs. 3I and 4I). Binding specificity was

**Fig. 1** Purification of the COOH-terminal region of CPB of *Leishmania (L.) amazonensis* (rCyspep). Electrophoresis assays were performed to assess the rCyspep protein (10  $\mu$ g) profile in denaturant conditions (a and b), non-reducing condition (c), and in native conditions (d), showing only one protein profile of 14 kDa and net charge. Molecular mass standard proteins (kDa) for SDS-PAGE and the PAGE path (-/+) are indicated on the left





**Fig. 2** Analysis of the interactions between the anti-rCyspep IgG and rCyspep protein. These experiments were performed with various concentrations of the rCyspep protein (a) on a COOH1 chip, which was coated with a specific anti-rCyspep IgG antibody. The data for the

dissociation resonance units (RU<sub>d</sub>) and protein concentration (µg) were analyzed by linear regression (b). These data are representative of three experiments

assessed using BSA as a negative control in a constant flow rate, which exhibited no non-specific interactions (Supp. 6A).

Taking the RU<sub>d</sub> values as effective interaction, it was possible to infer the amount of reactive protein with the antibody in the protein fractions of promastigotes and amastigotes. The SPR data were analyzed by linear regression including five concentrations of tested proteins (data not shown), in which the protein mass determination coefficients able to induce a response on the sensor ( $R^2$ ) indicated an antibody concentration-dependent binding tendency, as follows: promastigotes (soluble proteins of culture supernatant ( $R^2 = 0.84$ ), membrane ( $R^2 = 0.93$ ), and flagellum ( $R^2 = 0.87$ ) fractions) and amastigotes (soluble proteins of culture supernatant ( $R^2 = 0.93$ ), membrane ( $R^2 = 0.70$ ), and flagellum ( $R^2 = 0.98$ ) fractions). Thus, smaller  $K_{dec}$  values were obtained for the membrane and flagellum fractions from both parasite forms, indicating a lower detection by the anti-rCyspep IgG antibody in these fractions. Additionally, the membrane protein fractions from amastigotes exhibited  $K_{dec}$  values that were approximately 10-fold lower (Table 1). Moreover, higher RU<sub>d</sub> values were related to the supernatants of promastigote and amastigote cultures, and these preparations presented the lowest amount of proteins bound to the anti-rCyspep IgG antibody due to the larger chip saturation constant (Table 1).

### Potential binding of E-64 to the proteins recognized by anti-rCyspep IgG

SPR assays were performed to assess the potential of the anti-rCyspep IgG antibody to recognize and interact with the parasite proteins by binding to E-64, a classic irreversible inhibitor of CP. Therefore, it was possible to infer whether the Cyspep detected was alone or with the unprocessed CPB form in the culture supernatant, membrane, or flagellum of the parasite.

According to the results, the immune complex formed on the surface of the chip was able to bind to E-64 with RU values of effective interaction similar to some of the tested protein preparations (Figs. 3II and 4II). The RU signal for the flagellum protein fraction and the culture supernatant remained in the range of  $52 \pm 8.7$  to  $40 \pm 2.9$ , whereas the RU signal for the membrane fractions was in the range of  $14.0 \pm 1.1$  to  $10.0 \pm 1.5$  for both promastigotes and amastigotes (Table 2).

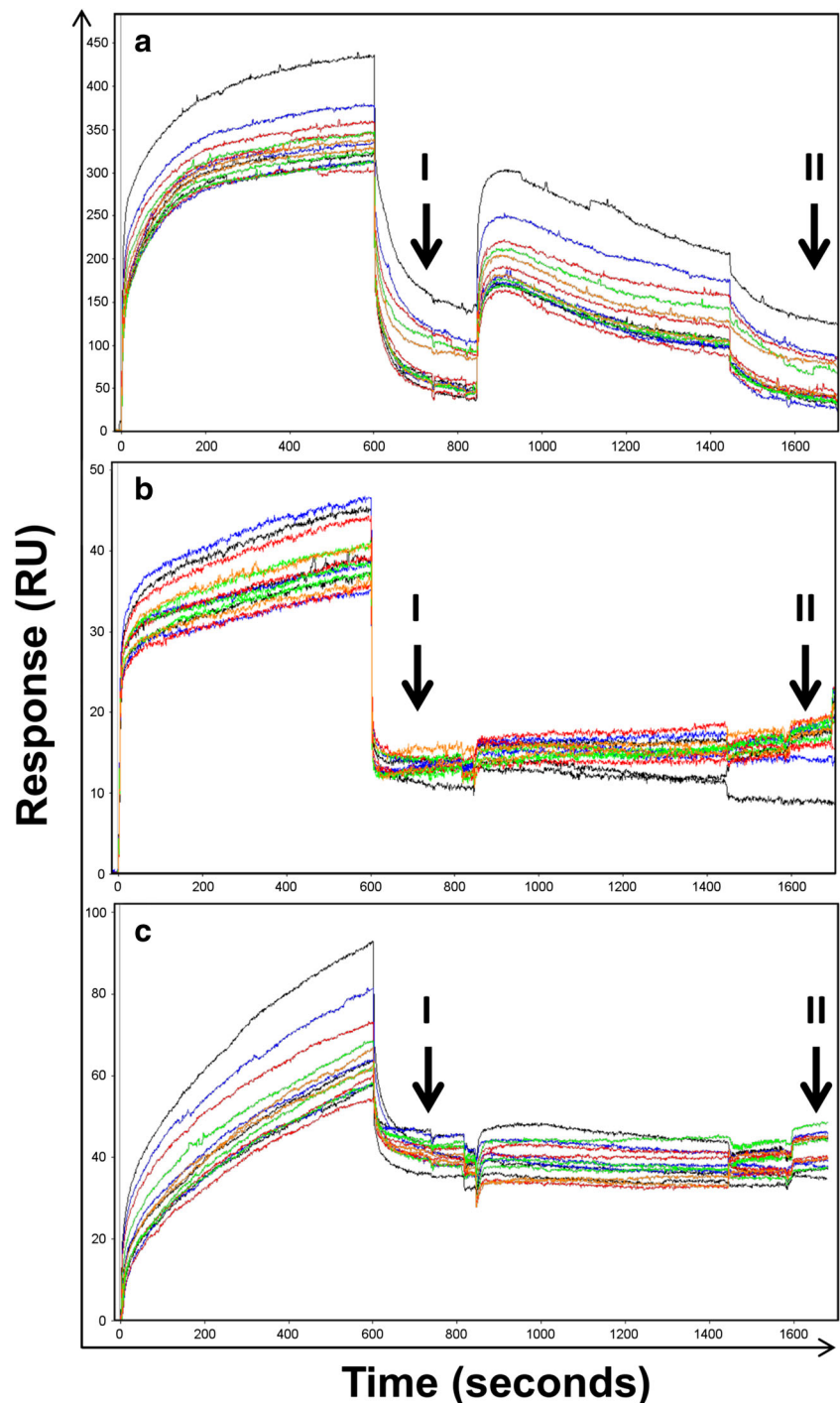
Although these results do not indicate a tendency of binding to E-64 in a dose-response curve of resonance, it was possible to detect a RU<sub>E-64</sub> signal higher than the RU signal of the complex formation between anti-rCyspep IgG and the protein preparations of promastigotes (1.4-fold for membrane fraction and 1.3-fold for culture supernatant) and amastigotes (1.3-fold for flagellar fractions) (Table 2). Thus, it was possible to infer that the amount of protein bound to the antibody, which retained the property of binding to E-64, was most strongly detected in the supernatant of the promastigote culture preparations ( $1.4 \times 10^{-2}$  ng/mm<sup>2</sup>) followed by the promastigote and amastigote flagellar and membrane ( $0.2 \times 10^{-2}$  ng/mm<sup>2</sup>) fractions and the amastigote membrane fraction ( $0.1 \times 10^{-2}$  ng/mm<sup>2</sup>).

These data were compared with control values in which the rCyspep anti-IgG complexes were subjected to SPR assays with various concentrations of E-64. Changes in the resonance signal were not detected in the presence of E-64, indicating no binding between the anti-rCyspep IgG antibody and the cysteine proteinase inhibitor (Supp. 6B).

### Discussion

The adaptation of *Leishmania* spp. to specific environments is associated with their differentiation, which occurs when the parasite alternates between the mammalian and sandfly hosts,

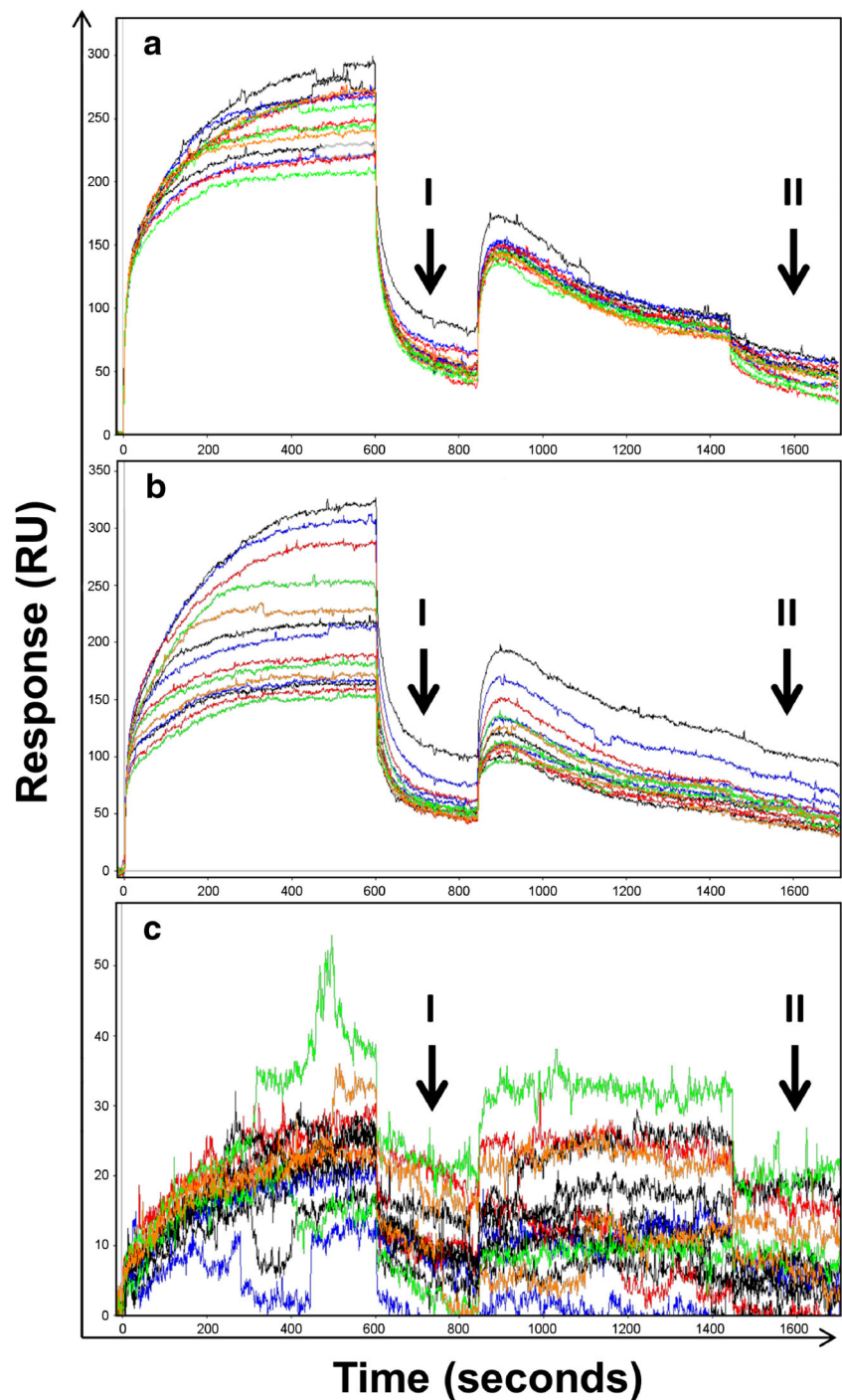
**Fig. 3** Sensorgrams for the detection of E-64 binding to promastigote proteins. After coating the COOH1 chip with the specific anti-rCyspep IgG antibody, the parasite proteins (culture supernatant (a), membrane (b), and flagellum (c) fractions) were captured (I) and subjected to a cycle of interaction with various concentrations of E-64 (— 10.0  $\mu$ M, — 1.0  $\mu$ M, — 0.1  $\mu$ M, — 0.01  $\mu$ M, — 0.001  $\mu$ M), for which the interaction (II) is described as signal resonance (RU). The graphs indicate the anti-rCyspep IgG–soluble Cyspep interaction profiles, as represented by the RU over time (seconds) of a representative experiment of three independent assays



and the expression of virulence factors, which include CPs. In this context, the release of Cyspep by the parasite into the new environment may contribute to this adaptive process in the host. Our results provide evidence that both the amastigote and promastigote forms of *L. (L.) amazonensis* release Cyspep. However, promastigotes release the polypeptide as unprocessed CPB, whereas amastigotes release the free polypeptide.

The approach employed in this study for Cyspep location was based on the ability to track this protein in the supernatant of the parasite culture on the parasite surface and the flagellar membrane-enriched fractions using a polyclonal antibody as a protein-capture tool through the SPR method. This approach was advantageous, since it enabled the analysis of binding phenomenon directly in complex environments as a mixture of proteins, in real time, considering the fourth dimension for

**Fig. 4** Sensorgrams for the detection of E-64 binding to amastigote proteins. After coating the COOH1 chip with the specific anti-rCyspep IgG antibody, the parasite proteins (culture supernatant (a), membrane (b), and flagellum (c) fractions) were captured (I) and subjected to interaction cycles with various concentrations of E-64 (— 10.0  $\mu$ M, — 1.0  $\mu$ M, — 0.1  $\mu$ M, — 0.01  $\mu$ M, — 0.001  $\mu$ M), for which the interaction (II) is described as signal resonance (RU). The graphs indicate the E-64–Cyspep interaction profiles, as represented by the RU over time (seconds) of a representative experiment of three different assays



molecular interaction (Van Regenmortel 1996). Therefore, the SPR system enables the study of dynamic protein-protein interaction reliably and not as a static phenomenon. Using this technique, it was possible to detect the formation of a complex between the anti-rCyspep IgG antibody and the Cyspep released in the supernatant of the parasite culture and the presence of Cyspep in either the surface membrane- and flagellum-enriched fractions of *L. (L.) amazonensis*.

Although the detection of immune complexes indicates the subcellular localization of Cyspep, our results do not confirm that this protein integrates into the parasite membrane. However, previous findings suggest that the CPs of *L. (L.) amazonensis* are located on the parasite surface membrane (Alves et al. 2000). Furthermore, it was also shown that CPB homologs are located on the surface of *L. (V.) braziliensis*, anchored by glycosylphosphatidylinositol



**Table 1** Quantitative analysis of *Leishmania (L.) amazonensis* protein detection by anti-rCyspep IgG

	Protein fractions	<sup>a</sup> RU <sub>d</sub>	Protein (ng/mm <sup>2</sup> )	<sup>b</sup> K <sub>dec</sub> (×10 <sup>-2</sup> μg)
Promastigote	Membrane fraction	61.0 ± 5.0	0.06 ± 0.005	6.0 ± 0.5
	Flagellum fraction	74.6 ± 6.0	0.08 ± 0.006	4.0 ± 0.4
	Culture supernatant	142.6 ± 15.0	0.14 ± 0.015	12.0 ± 1.2
Amastigote	Membrane fraction	48.0 ± 6.0	0.05 ± 0.006	0.4 ± 0.06
	Flagellum fraction	36.7 ± 4.0	0.04 ± 0.004	6.0 ± 0.40
	Culture supernatant	199.4 ± 2.0	0.20 ± 0.020	13.0 ± 1.3

<sup>a</sup> Dissociation resonance unit (RU<sub>d</sub>)<sup>b</sup> Constant of detection (K<sub>dec</sub>)

(Rebello et al. 2009). Further studies are required for a detailed understanding of the biomolecular interaction of Cyspep in the membrane surface of *L. (L.) amazonensis*.

There have been 87 families of CPs identified based upon the composition of their structural organization (<http://merops.sanger.ac.uk/>). E-64 is a specific irreversible inhibitor of CPs, such as cathepsins (K, B, and H), actinidin, and calpain (Lecaille et al. 2002). However, certain CP orthologs, such as bromelain, are poorly inhibited by E-64 (Harrach et al. 1998), whereas others, such as falcipain 1, are not inhibited (Goh et al. 2005) by E-64. Certain CP isoforms can interact covalently with E-64, while others may not. As Cyspep is exclusively a CPB enzyme, the capture of this protein by polyclonal antibodies ensures its presence and the property of binding E-64 of CPB in parasite fractions (surface and flagellar membranes).

This fact was demonstrated here by the association of sub-cellular fractionation by a surface biosensor approach. This strategy was important for demonstrating that amastigotes and promastigotes release Cyspep into the extracellular medium in distinct ways. We provide evidence that Cyspep from promastigotes can be secreted into the extracellular medium as the unprocessed enzyme CPB, suggesting that enzyme processing into the mature form occurs outside of the intracellular environment. This finding suggests that CPBs released by

promastigotes remain in the unprocessed form to participate in an as yet unknown biological event.

Antimicrobial peptides from bacteria (Pütsep et al. 1999) and higher organisms (Park et al. 1998) can be produced from the degradation of larger proteins. In general, bacteria use a variety of mechanisms to regulate and produce active peptides and proteins. Most toxins from bacteria are produced as precursor peptides that are modified post-synthesis inside or outside of the cell. The export of proenzymes and their subsequent processing into biologically active forms has been identified as a defense mechanism against tumors and pathogens (Deng et al. 2017). The extracellular processing of proteins may generate antimicrobial peptides, as described in *Propionibacterium jensenii* (Faye et al. 2002). Therefore, the extracellular processing of pro-CPB by promastigotes may be interpreted as a strategy of *L. (L.) amazonensis* to generate Cyspep that is structurally stable to guarantee its biological activities in the external environment.

Additionally, most of proteins that have a biological function in the extracellular environment contain disulfide bonds, which influence the thermodynamics of protein folding, stabilize the native conformation, and maintain the integrity of front protein oxidizing agents and proteolytic enzymes in the extracellular environment. Disulfide bonds can stabilize the protein structure by protecting the protein from damage and

**Table 2** Quantitative analysis of E-64 detection binding proteins of *Leishmania (L.) amazonensis*

	Protein fractions	<sup>a</sup> RU <sub>d</sub>	<sup>b</sup> ng/mm <sup>2</sup> (×10 <sup>-2</sup> )	<sup>c</sup> RU <sub>d E-64</sub>	<sup>b</sup> ng/mm <sup>2</sup> (×10 <sup>-2</sup> )	<sup>d</sup> RU <sub>d E-64</sub> - RU <sub>d</sub>	<sup>b</sup> ng/mm <sup>2</sup> (×10 <sup>-2</sup> )
Promastigote	Membrane fraction	14.0 ± 1.1	1.4 ± 0.1	16.0 ± 1.1	1.60 ± 0.01	2.0 ± 1.1	0.20 ± 0.11
	Flagellum fraction	40.0 ± 2.9	4.0 ± 0.3	40.6 ± 0.5	4.00 ± 0.05	0.0 ± 0.0	0.00 ± 0.00
	Culture supernatant	49.0 ± 5.7	4.9 ± 0.6	63.0 ± 6.8	6.3 ± 0.098	14.1 ± 1.1	1.4 ± 0.11
Amastigote	Membrane fraction	10.0 ± 1.5	1.0 ± 0.06	09.0 ± 1.5	9.0 ± 0.0015	1.0 ± 1.5	0.10 ± 0.15
	Flagellum fraction	52.0 ± 8.7	5.2 ± 0.09	54.0 ± 9.2	5.4 ± 0.009	2.0 ± 1.5	0.20 ± 0.15
	Culture supernatant	50.0 ± 2.5	5.0 ± 0.02	50.0 ± 2.8	5.0 ± 0.003	0.0 ± 0.0	0.00 ± 0.00

<sup>a</sup> Dissociation resonance unit (RU<sub>d</sub>)<sup>b</sup> Quantity of protein on the chip<sup>c</sup> RU<sub>d</sub> of E-64 binding (RU<sub>d E-64</sub>)<sup>d</sup> RU<sub>d E-64</sub> - RU<sub>d</sub>

increasing its half-life (Hogg 2003). The Cyspep protein has nine cysteine residues, which have the potential to form disulfide bonds (Alves et al. 2001a, b; Santos et al. 2016; Souza-Silva et al. 2014), supporting the hypothesis that Cyspep would maintain its structural integrity outside of the parasite.

Furthermore, Cyspep was detected in the amastigote culture supernatant, which supports the finding that this protein modulated the immune response elicited by *L. (L.) amazonensis* during an *in vivo* experimental infection using BALB/c. In macrophages, amastigote Cyspep is released during the maturation of pro-CPB and is directed to intracellular processing pathways of the mouse MHC system. Taken together, these findings suggest that the parasite may use protein processing products to establish an infection in the vertebrate host by modulating the type 1 or type 2 immune response (Alves et al. 2004; Pereira et al. 2011; Souza-Silva et al. 2014; Da Silveira-Júnior et al. 2017).

## Conclusion

The expression of virulence factors, such as CPB and its products, in the host cells suggests an evolutionary feature of *L. (L.) amazonensis* that contributed to shaping the lifestyle of this parasite. Here, we demonstrate that the COOH-terminal region of the CPB protein has a distinct fate in each form of the parasite. Using the following sequential steps, the Cyspep polypeptide was detected in both amastigotes and promastigotes: (i) antibody immobilization on a COOH1 chip followed by interaction with (ii) parasite proteins and then with (iii) E-64 for SPR. Both parasitic forms yielded Cyspep in the extracellular environment, but only promastigotes yielded this polypeptide as the unprocessed CPB enzyme.

**Acknowledgments** We are grateful to the facilities and technical support of the Fundação Oswaldo Cruz Technological Platforms, the Surface Resonance Plasmonic Platform (RPT03E), Real-Time PCR-RJ (RPT09A), Multiuser Research Facility of Flow Cytometry-Parametric Analysis (IOC), and Culture Medium Platform (IOC).

**Funding information** This study was financed in part by the Conselho Nacional de Desenvolvimento Científico e Tecnológico—Brasil (CNPq) (509737/2010-2; 401356/2014-0), the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro—Brasil (FAPERJ) (E-26/102.413/2010; E-26/111.954/2011; E-26/110.592/2012), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001. Bernardo A. S. Pereira and Francisco O.R. Oliveira Junior are research fellows of Fiocruz. Carlos R. Alves and Mirian C.S. Pereira are research fellows of the CNPq institution.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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