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Research paper

Is the activity of CGRP and Adrenomedullin regulated by RAMP (−2) and (−3) in Trypanosomatidae? An *in-silico* approach

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ABSTRACT

The Calcitonin-Like Receptor (CLR) belongs to the classical seven-transmembrane segment molecules coupled to heterotrimeric G proteins. Its pharmacology depends on the simultaneous expression of the so-called Receptor Activity Modifier Proteins (RAMP-) −1, −2 and −3. RAMP-associated proteins modulate glycosylation and cellular traffic of CLR, therefore determining its pharmacodynamics. In higher eukaryotes, the complex formed by CLR and RAMP-1 is more akin to bind Calcitonin Gene-Related Peptide (CGRP), whereas those formed by CLR and RAMP-2 or RAMP-3, bind preferentially Adrenomedullin (AM). In lower eukaryotes, RAMPs, or any homologous protein, have not been identified until now. Herein we demonstrated a negative chemotactic response elicited by CGRP (10^{-9} and 10^{-8} M) and AM (10^{-9} to 10^{-5} M). Whether or not this response is receptor mediated should be verified, as well as the expression of a 24 kDa band in *Leishmania*, recognized by western blot analysis by the use of (human-)-RAMP-2 antibodies as detection probes. Queries with human RAMP-2 and RAMP-3 protein sequences in blastp against *Leishmania* (*Viannia*) *braziliensis* predicted proteome, allowed us to detect two sequence alignments in the parasite: A RAMP-2-aligned sequence corresponding to *Leishmania* folylpolyglutamate synthase (FPGS), and a RAMP-3 aligned protein, a hypothetical *Leishmania* protein with yet unknown function. The presence of homologous of these proteins was described *in-silico* in other members of the *Trypanosomatidae*. These preliminary and not yet complete data suggest the feasibility that both CGRP and Adrenomedullin activities may be regulated by homologs of RAMP- (−2) and (−3) in these parasites.

1. Introduction

The Calcitonin peptide family include calcitonin (CT), Calcitonin Gene-Related Peptide (CGRP) -α and -β, Adrenomedullin (AM) and Amylin (AMY). These molecules share structural and functional homologies. Their activity produces anti-inflammatory and vasodilatory effects, as well as specific gastrointestinal effects on multicellular eukaryotes (Barwell et al., 2011; Bower and Hay, 2016), suggesting that their receptors play fundamental roles and may constitute potential pharmacological targets. Even more, beta-amyloid (Abeta) related activity of AMY has been associated with Alzheimer's disease (Mietlicki-Baase, 2017).

As described in Fig. 1, two calcitonin peptide sub-families interact with two different G-protein coupled receptors (GPCRs): the calcitonin-receptor like receptor (CRLR, Fig. 1A) and the calcitonin receptor (CTR, Fig. 1B) (Barwell et al., 2011). Their association with Receptor Activity Modifying Proteins (RAMP-) 1, 2, or 3 in humans (McLatchie et al., 1998; Parameswaran and Spielman, 2006) indicate a high level of sophistication that translates into (a) fine modulation of receptor selectivity towards the different calcitonin peptides and (b) triggering of various mechanisms of action depending on which G-protein system is activated. In fact, except for the case of CT, the receptor complex is a heterodimeric protein formed by a GPCR and either one of the RAMPs. RAMPs constitute a family of only one transmembrane segment proteins which achieve roles like allosteric modulators of molecular folding and cellular traffic, and promote glycosylation on the associated GPCR thus conferring unique effector functions (Gingell et al., 2016).

When the CRLR protein associates with RAMP-1, the CGRP receptor arises, whereas CRLR association with either RAMP-2 or RAMP-3 results in AM- type 1 and 2 receptors, respectively (Hinson et al., 2000, see Fig. 1A). In the case of CTR, association with either RAMP results in AMY receptors (Barwell et al., 2011; see Fig. 1B). Besides ligand binding, receptor trafficking and desensitization, expression regulation of RAMPs and GPCRs modulate down-stream signaling pathways, thus

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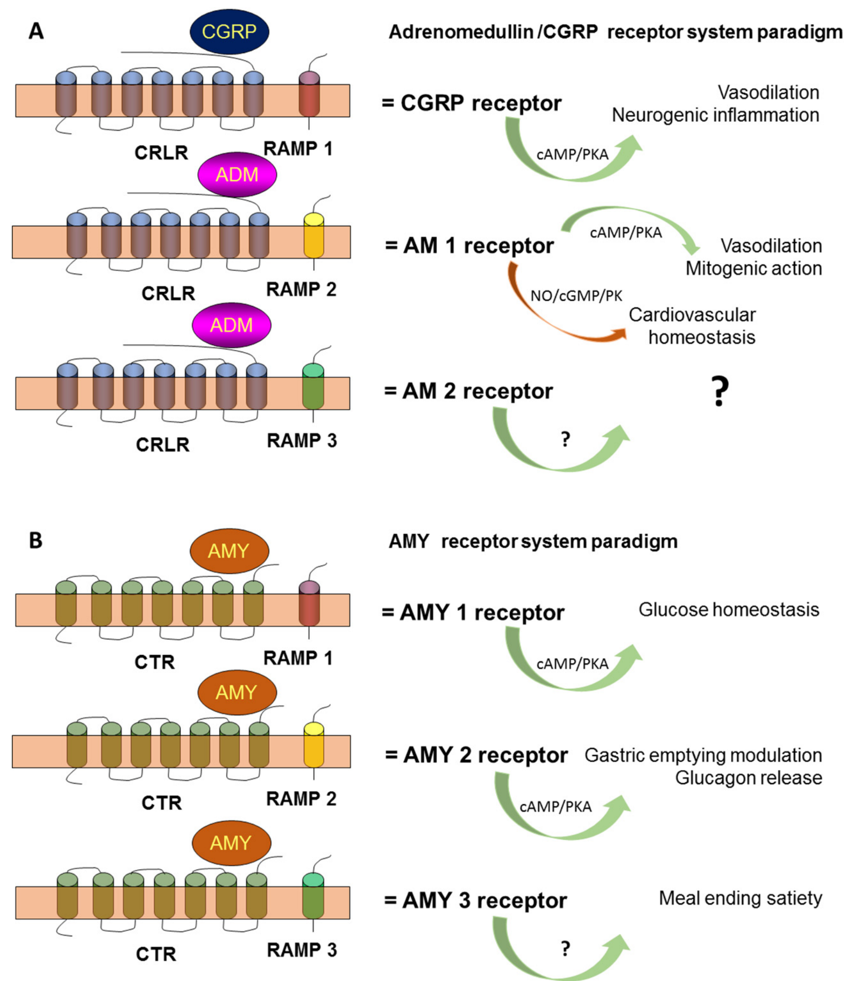

Fig. 1. Schematic diagram of CRLR (A) and CTR (B) association with RAMP-1 (orange), RAMP-2 (yellow) and RAMP-3 (green) to conform the different G protein receptors subtypes and their functions. AM, adrenomedullin; AMY, amylin; cAMP, cyclic AMP; cGMP, cyclic GMP; CGRP, calcitonin gene related peptide; NO, nitric oxide; PK, protein kinase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

altering receptor responsiveness, cellular phenotype and triggered physiological functions (Klein et al., 2016, see Fig. 1 for examples).

Due to their main effects as modulators of GPCRs, RAMPs can be defined as molecular chaperones. RAMP-interacting GPCRs identified until now include families A, B and C (Klein et al., 2016). Other GPCR members of the secretin receptor family (Archbold et al., 2011) also interact with RAMPs; however, their functional significance is currently unknown.

Although RAMP expression is common among multicellular eukaryotes, and experimental evidence traces RAMP expression back to simple organisms such as the zebrafish, their presence has not been acknowledged in unicellular eukaryotes. Notwithstanding, G protein signaling pathways have been described in unicellular parasites such as *Plasmodium falciparum* (Kaiser et al., 2015; Krai et al., 2014), but not yet in *Leishmania* or additional members of the Trypanosomatidae. Their portrayal would be fundamental since pharmacological intervention of conserved G protein signaling pathways might help to understand the physiology of these pathogens and may constitute targets for tool design against diseases caused by them.

Leishmania parasites are responsible for diseases globally termed leishmaniasis. Their clinical manifestations depend both on the infecting parasite species and on the host immune response. Limited but disfiguring skin infections are called cutaneous leishmaniasis. Upon spreading to spleen and liver, visceral leishmaniasis arises, a lethal if untreated disease characteristic of *Leishmania (L.) donovani* infection. Still, it has been described as associated with other *Leishmania* species (Goto and Lauletta Lindoso, 2012; van Griensven and Diro, 2012).

Leishmania membrane proteins accomplish fundamental roles including the primary function of sensing minimal changes in their milieu and working as a communication interface between extracellular and intracellular settings, with the goal of preserving integrity and survival through the parasite life cycle and during host invasion. Chemotaxis is an expression of the value of communication between cells to tune their position up in certain environments. It guarantees successful host cell-parasite interactions, and cell-invasion.

The report of any of the GPCR-related signal transduction systems (e.g. heterotrimeric G-proteins, 7-transmembrane receptors, effector proteins, RAMPs) has been elusive in members of the *Trypanosomatidae* including *Leishmania* parasites. Our data suggest a negative chemotactic response elicited by CGRP (10^{-9} and 10^{-8} M) and AM (10^{-9} to 10^{-5} M). Whether or not this effect is receptor mediated remain as an open question, as well as the involvement of second messengers linked to G proteins, as has been described in unicellular organisms like *Tetrahymena* (Kőhidai et al., 2016). Additionally, hereby we present for the first time, hints of the existence of a RAMP-2 antigenically related protein in *Leishmania*, as well as in silico clues of the existence of homologs of this protein and RAMP-3 in *Leishmania*. These preliminary data demonstrate the possibility of the existence of GPCRs for CGRP and AM in *Leishmania* that could be regulated by RAMP- (−2) and (−3) homologs.

2. Materials and methods

2.1. Parasite culture

2.1.1. Strain and culture conditions

Reference strains *Leishmania* (*V.*) *braziliensis* (MHOM/BR/LTB300) and *Leishmania* (*L.*) *mexicana* (MHOM/BR/82/Bel21) were kindly provided by Dr. Noris Rodríguez (Universidad Central de Venezuela); *Leishmania* (*L.*) *amazonensis* (MHOM/BR/77/LTB0016) was kindly donated by Dr. Lionel Schnur (University of Jerusalem). Parasites isolated from lesions were obtained from three patients suffering Diffuse Cutaneous Leishmaniasis (DCL) that regularly attended the Dermatology Department, Institute of Biomedicine, MPPS-UCV, Venezuela. The patients did not react to the treatment and had frequent relapses with Glucantime®. The following codes were assigned to the isolates: *L. (L.) mexicana* MHOM/VE/1996/ZC (VE96ZC), *L.* (*L.*) *amazonensis* MHOM/VE/1998/MR (VE98MR) and MHOM/VE/2000/MM (VE2000MM) (Padrón-Nieves et al., 2014). To minimize phenotype changes, the parasites were maintained in liquid nitrogen until use. *Leishmania* promastigotes were grown at 26 °C in semisolid blood agar supplemented with glucose-NaCl medium (glucose 1.5%, NaCl 0.85%, w: v.

Promastigotes at late log growth phase were collected by centrifugation at 125 ×*g* for 10 min at room temperature (RT). The medium was decanted and the cells were suspended in buffer A: Hepes 10 mM pH 7.3; NaCl 132 mM; KCl 3.5 mM; $CaCl_2$ 1 mM and $MgCl_2$ 0.5 mM, 288 mOsm/kg. The cells were centrifuged again and the buffer was discarded; the cells were washed again twice using this same procedure, and finally were suspended in buffer A up to the desired cell density.

2.2. Western blot

Cell lysates were prepared from promastigotes of the described strains and isolates. The samples were separated in by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in 15% acrylamide gels. After separation, the protein bands were transferred to nitrocellulose membranes by semi-dry electro-transference, according to standard methods. Blocking of the membranes was achieved using 5% fat milk in TBST (Tris 100 mM, 0.9% NaCl, pH 7.4, and 0.5% tween 20) for at least 2 h. Then, the membranes were washed using TBST.

Immunoblotting was then performed, using anti-RAMP-1 [C-terminal, Sigma®, 1:1000] or anti-RAMP-2 [H139, Santa Cruz Technologies®, 1:500] commercial antibodies, sank in TBST for 2–18 h at 4 °C. Afterwards, washing was performed and secondary antibody coupled to horseradish peroxidase used thereafter at 1:5000, for 2 to 18 h at 4 °C. The antibody WIC 108.3 (internal control) binds to the lipophosphoglycan backbone of *Leishmania* promastigotes. Membrane development was performed after washing in TBST, using citrate-phosphate buffer (25.7 mL Na_2HPO_4 [0.2 M], 24.3 mL citric acid [0.1 M], 50 mL dH_2O, pH 5.00, and ortho-phenylenediamine [OPD], [10 mg/25 mL buffer]). The reaction was started using 400 μL H_2O_2 (3%) per 100 mL buffer, ran for at least 2 min, and stopped by adding dH2O. Relative signal of each band was measured using the software ImageJ.

2.3. Chemotactic effect of AM and CGRP on Leishmania spp.

The chemotactic response of *Leishmania* promastigotes to AM and CGRP was determined by a modification of the vertical two chamber capillary assay (Köhidai, 1995) as thoroughly described elsewhere (Díaz et al., 2011, 2015). Briefly, the tips of an 8-channel-micropipette were used as the inner chamber and the wells of a 96-well plate were used as outer compartments of the two-chamber system. The tips were filled with a solution containing the peptides (CGRP and AM 10^{-10} to 10^{-5} M) dissolved in buffer A (Hepes 10 mM pH 7.3; NaCl 132 mM; KCl 3.5 mM; $CaCl_2$ 1 mM and $MgCl_2$ 0.5 mM). Tip number one was used as control (untreated cells) and was filled with buffer A alone. The wells were filled with the *Leishmania* suspension (200 μL, 4×10^7 cells mL^{-1}) prepared in buffer A. The cells (200 μL of parasite suspension at a cellular density of 4×10^7 cells/mL) were incubated for 30 min at RT to guarantee chemotactic and not chemokinetic responses (Díaz et al., 2011, 2013).

At the end of the incubation time the cells that migrated into the inner chamber were fixed in 2% formaldehyde in PBS (phosphate buffer 0.05 M, pH 7.2; NaCl 0.9 M) and counted in a haemocytometer. Tip number one represents our control of migration toenumerate the number of cells that migrated into the inner chamber without any chemical influence (100% in the Y axes, untreated cells). All additional percentages of migrating cells are referred to this 100%. A positive control has been included in the experiments; as glucose has been demonstrated to induce chemotaxis in *Leishmania*, we included a tip in which we measured the number of cells that migrated into the inner chamber in the presence of glucose 25 mM (Diaz et al. 2011, 2013).

After chemotactic profile evaluation, the role of peptide receptors was analyzed. That is, 1 mL of parasite suspension (4×10^7 cells/mL) was incubated for 12 min with 1 mL of known antagonists to CGRP and AM at a concentration 2 orders of magnitude higher than the peptide concentration eliciting the best and significant chemotactic response; afterwards, the dual chamber experiment was performed to test the interference elicited by antagonists on the chemotactic response.

2.4. In silico characterization of proteins

2.4.1. Identification of Leishmania homologs

Amino acid sequences of the human RAMP- (−1), (−2), and (−3), CGRP-1 (α) and 2 (β), and CLR were retrieved from the UniProt database (accession numbers O60894, O60895, O60896, P06881, P10092, D3DPG9, respectively) and used as a query for a blastp search against *Leishmania* (*V.*) *braziliensis* predicted proteome. DELTA-BLAST protocol was used as it outperforms other blast algorithms in detecting alignments among highly divergent species (Boratyn et al., 2012), with the position-specific matrix (PSSM) BLOSUM62, a word size equal to 2, and expect threshold of 10. Those alignments with both an *E*-value < 1 and at least 30% of identity were selected for further analysis using the web-based software prss3, which employs the Smith-Waterman algorithm to score alignments between two sequences. Alignments determined to have an *E*-value of 10^{-3} or less by this method were used for further in silico analyses, which included detection of conserved protein domains in Pfam, (Finn et al., 2014) the prediction of transmembrane segments using the web-based tool TMpred, (Hofmann and Stoffel, 1993) and computing secondary protein structure through ab initio methods employing Phyre2. (Kelley et al., 2015). Amino acid sequences of RAMP-2 and RAMP-3 eukaryotic orthologs were retrieved from the NCBI Reference Sequence Database, and an alignment using these and the *Leishmania* chosen protein sequences was constructed in Clustal Omega, which then allowed estimation of the corresponding maximum parsimony tree in MEGA 7.0.21 and further alignment editing in JalView.

2.4.2. Trypanosomatidae orthologs

To identify related proteins in other Trypanosomatidae, a DELTA-BLAST search was performed adopting the formerly results against the predicted proteomes of those *Leishmania* species currently available. A similar method was followed to identify alignments in *Trypanosoma* and *Crithidia*. Afterwards, an alignment was performed processing these sequences in Clustal Omega, and later employed in MEGA 7.0.21 for maximum likelihood tree construction.

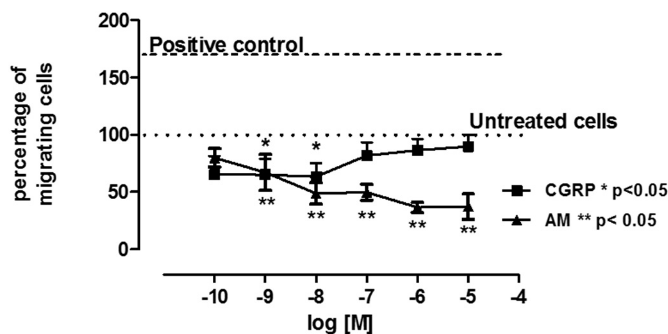

Fig. 2. Concentration dependence of chemotaxis elicited by CGRP and AM. *L. (V.) braziliensis* (LTB300) migration was significantly inhibited by CGRP (10^{-9} and 10^{-8} M) and by AM (10^{-9} to 10^{-5} M). Positive control = glucose (25 mM).

3. Results

3.1. Chemotactic effect of AM and CGRP

Neuropeptides may be fundamental signal molecules for the cell migration required during invasion and host-cell-parasite interaction by *Leishmania*. To explore if any of the GPCR-related signal transduction systems including heterotrimeric G-proteins, 7-transmembrane receptor or RAMPs related to taxis and migration are expressed in these parasites, we analyzed the effect of AM and CGRP on in vitro chemotaxis experiments. Fig. 2 describes the obtained results. Percentage of parasites migrating into the inner chamber in untreated conditions represents 100%. As glucose (25 mM) has been described to promote chemotaxis and migration in *Leishmania* parasites we used it as a positive control (Diaz et al. 2011, 2013). Both CGRP (10^{-9} and 10^{-8} M) and AM (10^{-9} to 10^{-5} M) produce a chemo-repellent effect on LTB300; i.e., a significant decrease in the percentage of parasites migrating into the inner chamber. Furthermore, Fig. 3 illustrates that the effect of CGRP, but not of AM, is antagonized by the truncated peptide $CGRP_{8–37}$.

However, the effect of AM is neither antagonized by the truncated peptide $AM_{22–52}$ nor by the antibody Anti-RAMP-2 at concentrations 100 to 1000-fold higher than those used to elicit the chemotactic effect. CGRP (10^{-8} M) neither affected the morphology (area) of the cell nor the length of the flagellum (data not shown). These results might suggest the absence of specific receptors for these neuropeptides in *Leishmania*; alternatively, they may also indicate the need to use more specific antagonists to determine the presence or not of the related receptors. However, additional analysis of the response is needed to find out the mechanism associated with this response.

3.2. Immunoblot identification of a RAMP- related epitope in Leishmania

To further analyze this chemotactic response, we performed a blot analysis of parasite homogenates. Fig. 4A illustrates how anti-RAMP-2, but not anti-RAMP-1 recognized epitopes in both reference *Leishmania* strains LTB0016 and LTB300. Furthermore, we identified a 24 kDa (Fig. 4B, molecular weight markers not included) RAMP-2 related epitope in the *Leishmania* reference strains LTB300, LTB0016, Bel21, as well as in isolates obtained from patients suffering DCL with chemotherapeutic failure against glucantime. As the results presented in Fig. 4B come from the same blot, with each lane containing comparable parasite material, we can conclude that in the isolates, expression of the novel epitope was dampened as compared to the reference strains.

3.3. Identification of h-RAMP-2 and h-RAMP-3 homologs in Leishmania

Results from the western blot analysis encouraged us to perform an *in-silico* study to determine the presence of this GPCR-related signal transduction system in *Leishmania*. In fact, a thorough DELTA-BLAST search of the predicted *L.* (*V.*) *braziliensis* proteome suggests the presence of multiple homologs with human RAMP- (−1), (−2), and (−3) (Table 1). Notwithstanding, the use of human CGRP-1 and -2 and CLR sequences, yielded no results using these nor other blast settings. A reciprocal best hit (RBH) approach displayed no homologs in neither case. After curating the results using prss3, two protein sequences, XP_001568902.1 (530 aa, previously confirmed as being folylpolyglutamate synthetase, Fig. 5A) (El Fadili et al., 2002) and XP_001566159.1 (226 aa, Fig. 5B), were selected for further analyses. In both cases, full-length sequences were used to build the alignments (see Fig. 5C).

To identify the ortholog candidates in other *Leishmania* species, *L.* (*V.*) *braziliensis* sequences were used as query sequences against predicted proteomes of Trypanosomatidae species, including *Trypanosoma* and *Crithidia*. The results suggest the existence of analogous sequences in *Leishmania* and *Trypanosoma*, but not in more divergent species as *Crithidia*, and *Sauroleishmania*. The alignments built by this analysis display a high degree of conservation, reason why *L.* (*V.*) *braziliensis* sequences were used for the remaining *in-silico* assays.

3.4. In silico characterization of Leishmania proteins

L. (*V.*) *braziliensis* aligned protein sequences used as queries in the Pfam database predicted the existence of a Mur ligase functional domain in the RAMP-2 homologous sequence, XP_001568902.1 (*E*-value = 3.9e−10, alignment length 192 amino acids, see Fig. 6 for a multiple alignment display). The results were negative for all other sequences. On the other hand, the web-based algorithm TMpred allowed the identification of five putative transmembrane helices in *L.* (*V.*) *braziliensis* XP_001568902.1 and two for the RAMP-3 homolog XP_001566159.1. Protein XP_001568902.1 was identified as the *Leishmania* enzyme folylpolyglutamate synthase (FPGS). Finally, prediction of the secondary structure of the candidate proteins was performed using the Phyre2 software (Fig. S1, supplementary material).

3.5. RAMP-2 and 3 alignments and phylogeny

An alignment was built in Clustal Omega using the sequences of RAMP-2 eukaryotic orthologs and the *L.* (*V.*) *braziliensis* protein XP_001568902.1, and RAMP-3 eukaryotic orthologs and the *L.* (*V.*) *braziliensis* protein XP_001566159.1. These alignments allowed the construction of a maximum parsimony tree (Fig. 7) in MEGA 7.0.21.

3.6. Trypanosomatidae homologs of the significant aligments

The sequences of *L.* (*V.*) *braziliensis* XP_001568902.1 and XP_001566159.1 allowed the identification of corresponding orthologs in several other members of the Trypanosomatidae family by a blast search. However, this approach yielded no results in the more divergent *Leishmania* and *Trypanosoma* species, or in the genus *Crithidia*.

Given the presence of homologs to these *L.* (*V.*) *braziliensis* proteins in several species of flagellates from the Trypanosomatidae family, a maximum likelihood tree was constructed to analyze the phylogenetic relationship among them, as shown in Fig. 8. All these proteins have conserved genomic contexts: For XP_001568902.1, its orthologs in *Leishmania* were located at chromosome 35 or 36, located downstream to the cyclin-e binding protein-1 like, and upstream to a putative protein kinase, as for *Trypanosoma*, the orthologs were located at chromosome 10, having a chromatin binding protein as a neighbor sequence. Additionally, this protein functions as a FPGS in *Leishmania*, and as a dual FPGS/Dihydrofolate synthase (DHFS) in *Trypanosoma*.

XP_001566159.1 orthologs, on the other hand, locate in chromosome 28 in the case of *Leishmania*, neighbor genes are copine-i-like protein and a putative glucose-regulated protein. In Trypanosoma, XP_001566159.1 orthologs locate neighboring the genes for the putative glucose regulated protein 78 and upstream to a vacuolar type H^+

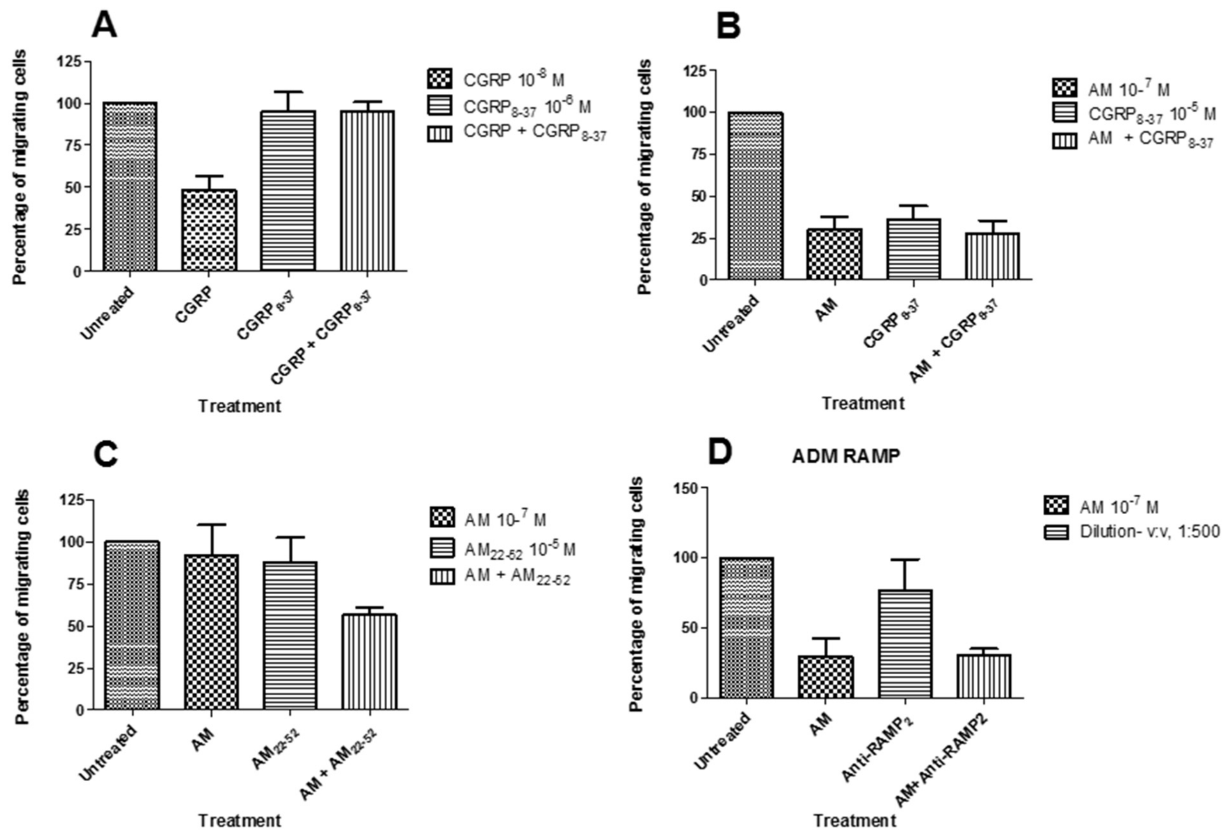

Fig. 3. Antagonism of $CGRP_{8–37}$ (A–B), $AM_{22–52}$ (C) and Anti-RAMP-2 (D) on chemotaxis elicited by CGRP and AM. The chemotactic effect of CGRP but not of AM is antagonized by the truncated peptide $CGRP_{8–37}$ and the effect of AM is neither antagonized by the truncated peptide $AM_{22–52}$ nor by Anti-RAMP-2 at concentrations 100 to1000-fold higher than those used to elicit the chemotactic effect.

ATPase subunit.

4. Discussion

Genetic divergence is remarkable when comparing Trypanosomatidae with other eukaryotes (El-Sayed et al., 2005). In Trypanosomatidae, shared genomic and evolutionary features is such that several proteins of one species have an ortholog in additional members of the family, resulting in only 5% of species-specific proteins. Despite this genomic background, it is uncertain whether or not regulatory mechanisms of cell signaling differ within Trypanosomatidae family members.

On the other hand, variations in intracellular messenger concentrations are triggered in response to minimal changes in physical and/or chemical signals occurring in the surroundings (Figarella et al., 2007). The events that traduce these environmental signs into activation or termination of intracellular signals fluctuations, as is the case for Ca^{2+} (Docampo and Huang, 2014) and cAMP (Biswas et al., 2011) are not defined for neither member of the Trypanosomatidae, and identification of the cellular mechanisms that result in their regulation, including the membrane transducers involved, have been elusive.

In fact, some membrane receptors, like an insulin-like growth factor receptor type I (Gomes et al., 2001), as well as putative adenylate-cyclase associated receptors (Sanchez et al., 1995) have been described in *Leishmania*. One may speculate about their role in life-cycle completion, such as nutrient acquisition (De Cicco et al., 2012; Krishnamurthy et al., 2005), or molecular monitoring of the extracellular media (Ghosh et al., 1996).

Furthermore, despite GPCR being ancient molecules, well known for switching cellular signaling in many contexts and organisms, their expression and of GPCR associated molecules and mechanisms has not been demonstrated in the Trypanosomatidae. Still, putative small GTPases (no true homologs of the human small GTPases) have been described in *T.* (*b.*) *brucei*, with homologs existing in *T. cruzi* and *L.* (*L.*) *major* (Field, 2005), accomplishing functions related to intracellular transport. In other cellular eukaryotes like *Tetrahymena*, majority of chemotactic ligands accomplish signaling via GPCR, although frequently their existence is only supported by circumstantial evidences like the effect of inhibitors (Kőhidai et al., 2016). In humans, AM activates a GPCR member of the secretin receptor family, a membrane heterodimeric protein complex formed by CLR and RAMP-2 (Parameswaran and Spielman, 2006).

Herein we show for the first time the chemotactic potency of AM and CGRP in *Leishmania*, being chemotaxis one of the essential migratory responses in these parasites, and provide for the first time, evidence of the expression of a *Leishmania* peptide antigenically identified by an antibody against RAMP-2 and therefore potentially related with a human GPCR.

In-silico analysis suggests the presence of homologs of the human RAMP- (−1), (−2), and (−3) in *Leishmania* as listed in Table 1. Interestingly, the use of human CGRP-1 and -2, and CLR sequences was not successful by means either of the blast settings used herein, nor other blast settings, and the same was true by a RBH approach. Nevertheless, after curating the results using the Smith-Waterman algorithm-based software prss3, two protein sequences, XP_001568902.1 and XP_001566159.1, yielded positive results good enough to be selected for further analyses. The Smith-Waterman algorithm (Smith and Waterman, 1981) estimates the probability of an alignment to be true following a more exhaustive, resource-demanding, mathematical approach in comparison to the heuristic methods used by BLAST (McGinnis and Madden, 2004).

Tertiary or quaternary structures are predicted using ab-initio

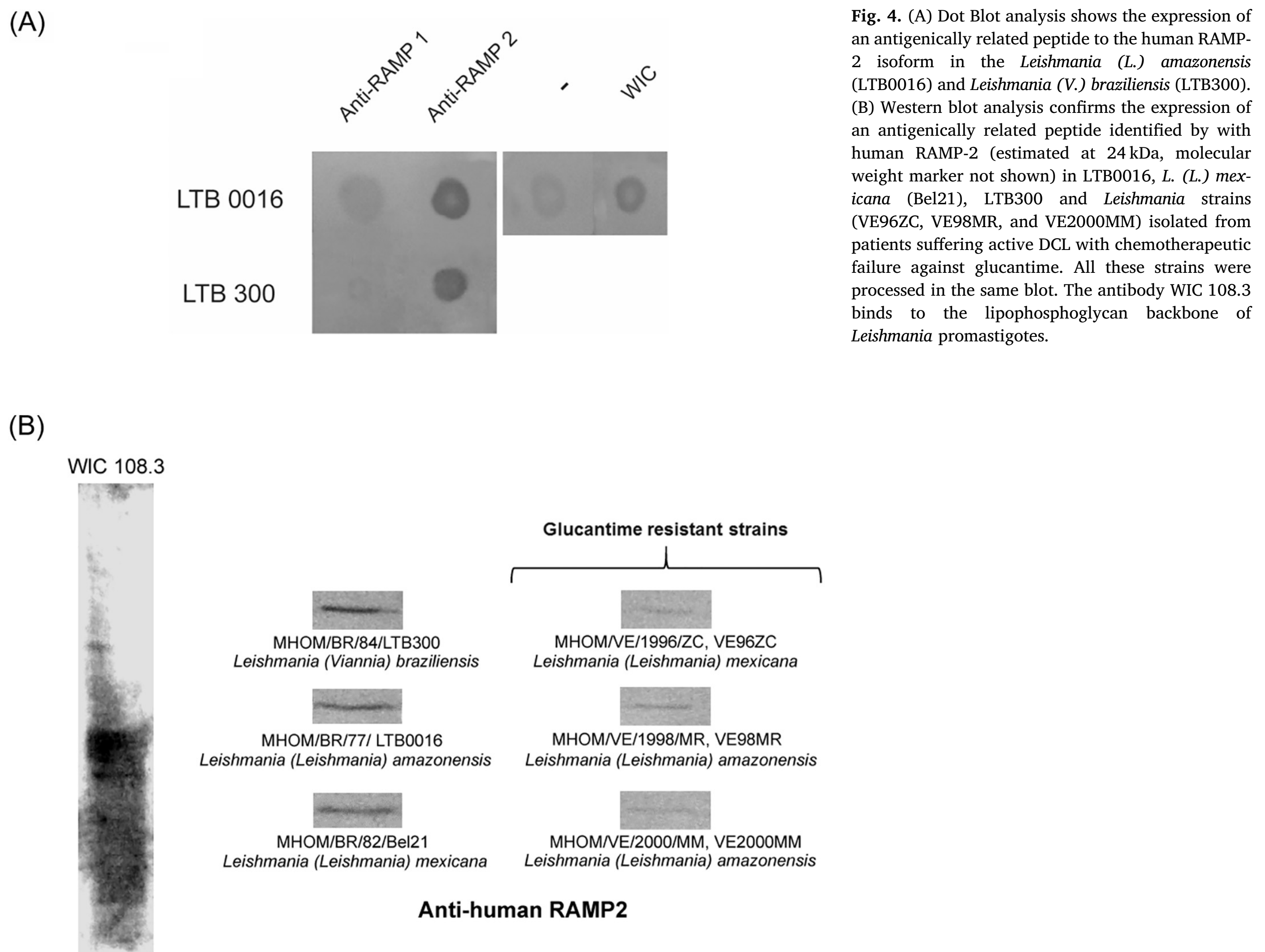

Fig. 4. (A) Dot Blot analysis shows the expression of an antigenically related peptide to the human RAMP-2 isoform in the *Leishmania (L.) amazonensis* (LTB0016) and *Leishmania (V.) braziliensis* (LTB300). (B) Western blot analysis confirms the expression of an antigenically related peptide identified by with human RAMP-2 (estimated at 24 kDa, molecular weight marker not shown) in LTB0016, *L. (L.) mexicana* (Bel21), LTB300 and *Leishmania* strains (VE96ZC, VE98MR, and VE2000MM) isolated from patients suffering active DCL with chemotherapeutic failure against glucantime. All these strains were processed in the same blot. The antibody WIC 108.3 binds to the lipophosphoglycan backbone of *Leishmania* promastigotes.

Table 1
Blast results of RAMP-1, 2, 3 alignments in *Leishmania* (*Viannia*) *braziliensis*.

Query Id	Accession number	Protein function	Identity percentage	*E*-value	Bit score	PRSS *E*-value	PRSS bit score
RAMP-1	XP_001568719.1	Conserved hypothetical protein	30.645	0.33	29.6	1.20E−02	30.9
	XP_001563418.1	Enolase	34.146	0.77	28.5	7.50E−02	27.6
RAMP-2	**XP_001568902.1**	**Folylpolyglutamate synthetase**	**30**	**0.48**	**29.6**	**1.70E−03**	**33.4**
	XP_001562684.1	Conserved hypothetical protein	36.957	0.99	28.5	1.00E−01	28
RAMP-3	XP_001568869.1	Putative developmentally regulated GTP-binding protein 1	31.25	0.52	28.9	2.10E−02	29
	XP_001566159.1	**Conserved hypothetical protein**	**30.909**	**0.64**	**28.5**	**1.40E−03**	**33**

Bold text identifies the protein sequences, XP_001568902.1 and XP_001566159.1 selected for further analyses.

methods; given the dimension of the herein presented proteins is mathematically laborious. However, α-helices and β-sheets could be distinguished from sequence analysis. The web-based algorithm TMpred allowed identification of five putative transmembrane helices in *L.* (*V.*) *braziliensis* XP_001568902.1 and two in the case of the RAMP-3 alignment XP_001566159.1. Moreover, XP_001568902.1 was identified as the *Leishmania* enzyme folylpolyglutamate synthase (FPGS) and XP_001566159.1 is tagged on databases as a hypothetical conserved protein. Finally, by the use of the software Phyre2, prediction of the secondary structure of candidate proteins was performed, and sizes surrounding 20 and 58 KDa were described for XP_001566159.1 and XP_001568902.1 respectively.

Folylpolyglutamate synthase (FPGS) has a central role in establishing and maintaining cytosolic and mitochondrial folylpolyglutamate concentrations in humans; thus, is essential for folate homeostasis and the survival of proliferating cells. *Leishmania tarentolae* FPGS gene has been isolated and characterized. Its predicted product contains 538 amino acids with 33 and 30% identity with the human and yeast FPGS proteins, respectively (El Fadili et al., 2002). The protein herein described is 518 amino acids long and has 30% identity with the human enzyme. FPGS activity is expressed in all forms of the parasite and modulates folate retention. Polyglutamylation may also be fundamental for folate compartmentalization within the cell (Vickers and Beverley, 2011). No role for this enzyme has been described in regard to the function of CGRP or AM.

Additionally, regardless of not having found any functional homolog to our hypothetical protein XP_001566159.1 through *in-silico* analysis, its size is similar to that described for mammalian RAMP-

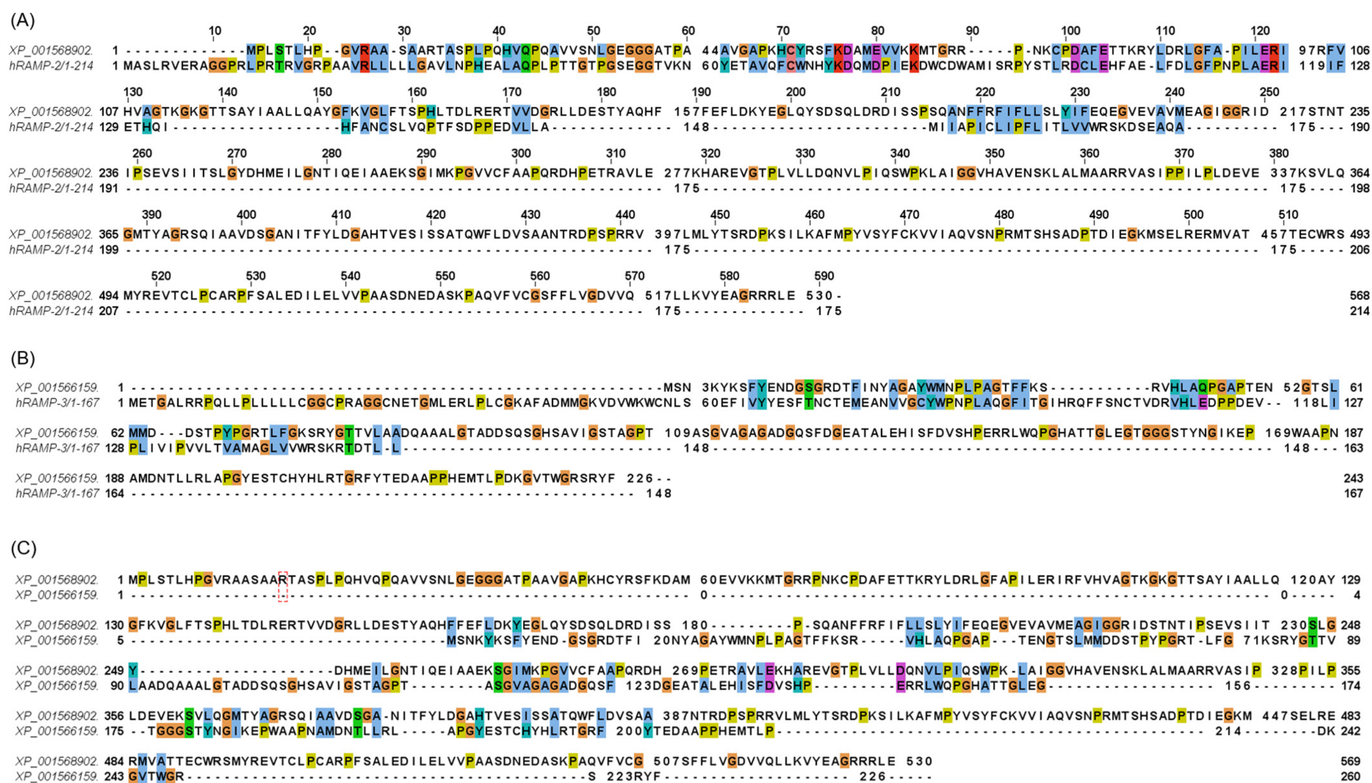

Fig. 5. Alignments built using (A) the human RAMP-2 and the *Leishmania (V.) braziliensis* protein XP_001568902.1, which is the enzyme folylpolyglutamate synthase (FPGS), (B) the human RAMP-3 and the *Leishmania (V.) braziliensis* XP_001566159.1, a hypothetical conserved protein with unknown function. In both cases, full-length sequences are used to build the alignments, and (C) the sequences XP_001568902.1 and XP_001566159.1 from *L.* (*V.*) *braziliensis*, which shows 22% identity between the sequences, with most of the aligned amino acids lying in the center of the *Leishmania* FPGS.

(−2) and (−3) (Zhao et al., 2006). Conservation of XP_001566159.1 and XP_001568902.1 in several *Leishmania* and *Trypanosoma* species suggests their link to essential Trypanosomatidae survival mechanisms. Indeed, sharing essential genes, unrelated to species-specific cellular mechanisms, characterize Trypanosomatidae (El-Sayed et al., 2005) although homologs of these proteins were not identified in neither *Crithidia* nor in the more divergent *Leishmania* species such as *L.* (*V.*) *lainsoni* or members of the subspecies *L. (Sauroleishmania)*. These results suggest a role for these proteins through the parasite life cycle either when they are prone to infect mammals, or for parasite survival within the vector or the host. It is noteworthy the decreased intensity of the band recognized in parasites isolated from DCL patients with chemotherapeutic failure against glucantime, when compared with reference *Leishmania* strains, underpinning the direct or indirect functions related to antimicrobial sensitivity, probably accomplished by the parasite hypothetic proteins.

Finally, it is thought-provoking that the extracellular portion of RAMP-2, presumably recognized by the anti-RAMP-2 antibody from humans used herein, has a region structurally similar to a protein apparently involved in chemotaxis in bacteria, the methyl accepting chemotaxis protein (Williams and Stewart, 1999). In microorganisms, this protein, as well as histidine kinases, are molecular devices for monitoring environmental changes. These molecules extracellular sensory domains are shared by different transmembrane receptors thus suggesting that similar conserved domains sense similar environmental signals and transmit information via different signal transduction pathways to different regulatory intracellular circuits, including chemotaxis (Bourret and Stock, 2002; Zhulin et al., 2003). Whether or not this is the case we are facing with, still needs to be elucidated.

Alignment of the sequences XP_001566159.1 and XP_001568902.1 shows an identity of around 22%. That, together with a highly-gapped alignment allow one to infer that these proteins are remotely related even within the same species. Despite the herein described relationship between these two proteins, both are conserved among different trypanosomatids. The genes that originate these molecules seem to have a common synteny within all the Trypanosomatidae species analyzed, which is a typical characteristic of these eukaryotic genomes (El-Sayed et al., 2005).

A maximum parsimony tree using the sequence XP_001568902.1, which is the *Leishmania* FPGS, and RAMP-2 shows that the protein shares the same clade with those mammalian RAMP-2 homologs, being more remotely related to reptile or fish proteins, and even less to that of birds. When comparing XP_001566159.1 with RAMP-3 eukaryotic homologs, the opposite relationship is found, where the leishmanial protein appears to be more closely related to those RAMP-3 fish homologs than those of mammalian species. Of note, the number of mutational events predicted to separate XP_001566159.1 to the apparently close fish RAMP-3 homologs is similar to that which separates XP_001568902.1 from the reptilian RAMP-2 homologs (Fig. 7). However, parsimony trees do not offer high reliability in inferring actual distances, but tree topology (Yang and Rannala, 2012).

A noteworthy fact is the similar topology predicted by the maximum likelihood trees of both proteins in Trypanosomatidae, that follows the taxonomic separation already agreed among Old World and New World *Leishmania*, as well as among *Leishmania* and *Viannia* subspecies, (Momen and Cupolillo, 2000) in this last case, sharing a predicted speciation event that early divided *L.* (*Viannia*) from other species from the same genus. Those more divergent *L.* (*Viannia*) and *L.* (*Sauroleishmania*) species lack an ortholog of neither of these proteins, raising the question of the need of this protein for the completion of the lifecycle of the parasite, leading us to hypothesize that its presence could relate with the epidemiological behavior of the disease.

Fig. 6. A multiple sequence alignment built from the *Leishmania (V.) braziliensis* protein XP_001568902.1 and several know *Klebsiella pneumoniae* and *Escherichia coli* muramyl ligases (MurC, MurD, MurE, MurF) is shown. Despite the sequence length difference, notice the presence of homologous regions interspersed within the sequence of the *Leishmania* protein which end up representing about a 40% similarity.

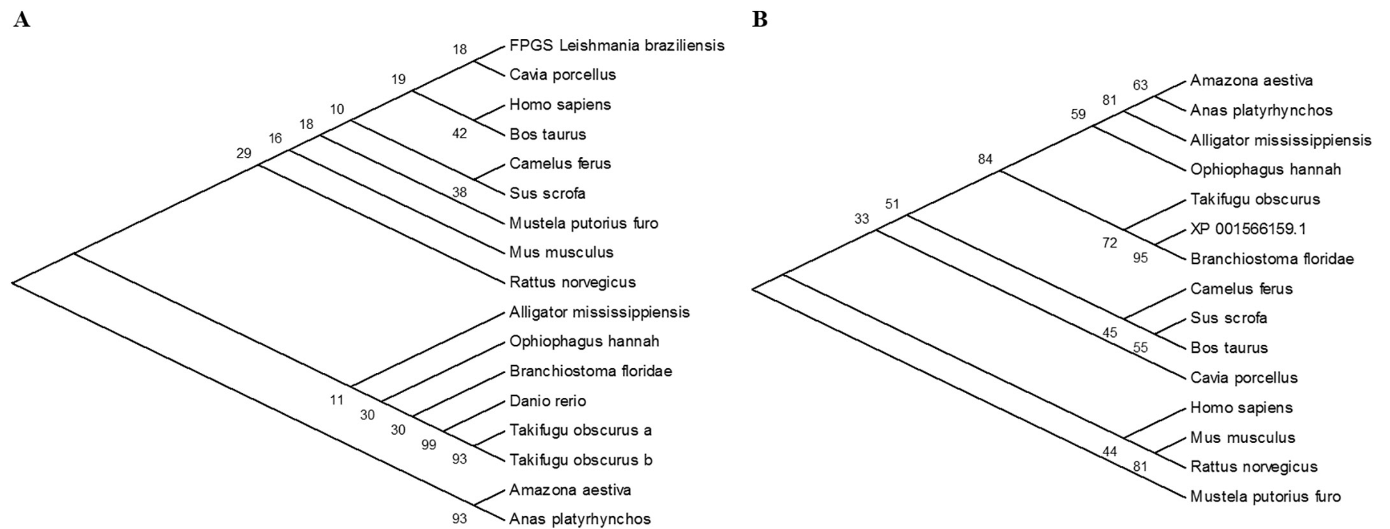

Fig. 7. Maximum parsimony consensus cladogram created using an amino acid substitution model and 500 bootstrap replications built using (A) an alignment between the *Leishmania* folylpolyglutamate synthase (FPGS) and the sequences for several eukaryotic RAMP-2 homologs. The topology of the tree shows a nearer relationship between the *Leishmania* FPGS and the *Carus porcellus* RAMP-2 ortholog compared to other homologs of the protein. (B) An alignment using the hypothetical protein `XP_001566159.1` and a set of RAMP-3 eukaryotic homologs shows a nearer phylogenetic relationship between the parasite protein and the RAMP-3 fish homologs.

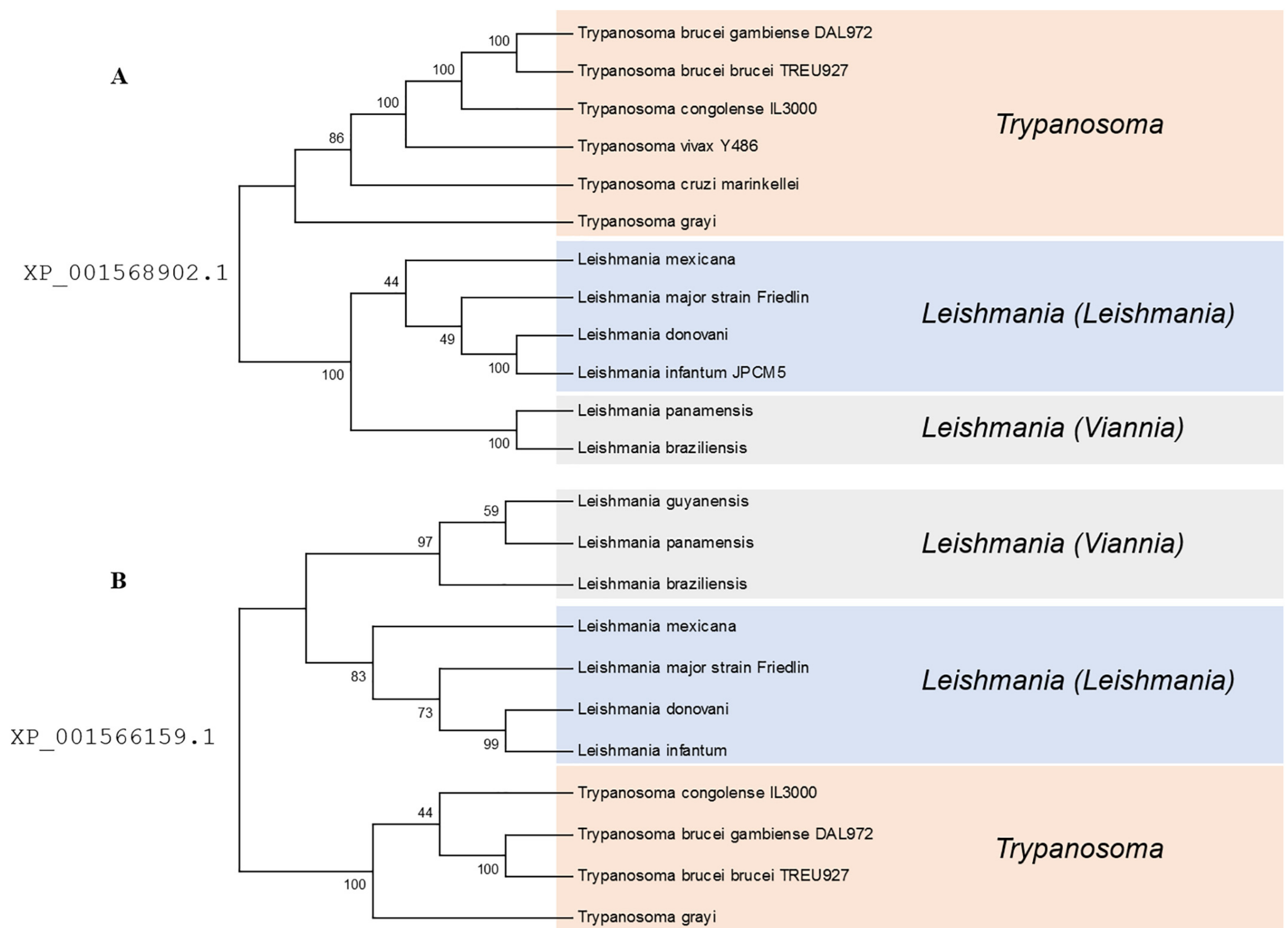

Fig. 8. Maximum likelihood cladogram, created using an amino acid substitution model and 500 bootstrap replications, employing alignments built from *Leishmania* and *Trypanosoma* FPGS (RefSeq: XP_001568902.1 for *L.* (*V.*) *braziliensis* homologs (A) and the hypothetical protein XP_001566159.1 (B)).

5. Conclusion

Herein we have described a negative chemotactic effect of the vertebrate/human-type vasoactive molecules AM and CGRP in *Leishmania*. Additionally, we have detected by immunoblot, and described by in silico tools, a putative RAMP-2 homolog in *Leishmania*. Both these findings suggest that molecules and protein(s) hereby proposed, involved in the associated receptor cascade might be present in lower eukaryotes, suggesting the ancient conservation of signaling systems associated with unicellular responses fundamental for cell survival, i.e., taxis and migration. This is a novel finding in parasite biology. Though, isolation and sequencing of the molecules involved in the response, and of the identified protein are necessary to further characterize them, structurally, genetically, and functionally. Notwithstanding, their conservation in Trypanosomatidae species, able to infect mammals, could carry a meaning to survival, revealing a possible therapeutic target for diseases caused by *Leishmania* and *Trypanosoma*.

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