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Molecular cloning, heterologous expression and functional characterization of a novel translationally-controlled tumor protein (TCTP) family member from *Loxosceles intermedia* (brown spider) venom☆

Youssef B. Sade[a,b], Marianna Bóia-Ferreira[a], Luiza H. Gremski[a], Rafael B. da Silveira[a,c], Waldemiro Gremski[a,d], Andrea Senff-Ribeiro[a], Olga M. Chaim[a], Silvio S. Veiga[a,*]

[a] *Department of Cell Biology, Federal University of Paraná, Curitiba, Paraná, Brazil*
[b] *Biotechnology Laboratory, National Institute of Metrology, Standardization and Industrial Quality (INMETRO), Duque de Caxias, Rio de Janeiro, Brazil*
[c] *Department of Structural, Molecular Biology and Genetics, State University of Ponta Grossa, Ponta Grossa, Brazil*
[d] *Catholic University of Paraná, Health and Biological Sciences Institute, Curitiba, Paraná, Brazil*

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ABSTRACT

Envenoming with brown spiders (*Loxosceles* genus) is common throughout the world. Cutaneous symptoms following spider bite accidents include dermonecrosis, erythema, itching and pain. In some cases, accidents can cause hypersensibility or even allergic reactions. These responses could be associated with histaminergic events, such as an increase in vascular permeability and vasodilatation. A protein that may be related to the effects of spider venom was identified from a previously obtained cDNA library of the *L. intermedia* venom gland. The amino acid sequence of this protein is homologous to proteins from the TCTP (translationally-controlled tumor protein) family, which are extracellular histamine-releasing factors (HRF) that are associated with the allergic reactions to parasites. Herein, we described the cloning, heterologous expression, purification and functional characterization of a novel member of the TCTP family from the *Loxosceles intermedia* venom gland. This recombinant protein, named LiRecTCTP, causes edema, enhances vascular permeability and is likely related to the inflammatory activity of the venom. Moreover, LiRecTCTP presents an immunological relationship with mammalian TCTPs.

1. Introduction

Brown spiders (*Loxosceles* genus) are found worldwide, and the prevalence of accidents with these animals has created a public health problem, especially in the south-central United States and in the southeast region of Brazil. Loxoscelism manifestations include necrotic lesions, gravitational spreading and a massive inflammatory response at the injured site. Lesions are normally preceded by edema, erythema and itching. In a lower percentage of cases, patients develop hematological dysfunctions and renal injury, which can evolve to renal failure and even lead to death (Kusma et al., 2008; da Silva et al., 2004). The venom has a complex molecular composition of several different toxins and is enriched with proteins that range from 3 to 40 kDa in molecular mass. The mechanisms by which the venom exerts its effects are still under investigation, although studies have shown that venom components act synergistically (da Silva et al., 2004; Appel et al., 2005). Phospholipases-D, which are major components of venom (also called dermonecrotic toxins), have been cloned and comprise a family of toxins present in several *Loxosceles* species (Chaim et al., 2006; Appel et al., 2008; da Silveira et al., 2006, 2007; Kalapothakis et al., 2007).

Histamine has been identified to be the principal pharmacological component in the venom of several spiders (Rash and Hodgson, 2002; Rash et al., 1998). The role of histamine and its receptors in the development of edema, increased vascular permeability and vasodilatation are considered relevant for loxoscelism (Rattmann et al., 2008). Histamine can also be released from mast cells in response to venom components. The presence of histamine in the envenomation site can cause pain, edema, erythema and endothelial changes such as increased vascular permeability and vasodilatation. These effects contribute to the systemic dispersion of the venom components (Mekori and Metcalfe, 2000; Weisel-Eichler and Libersat, 2004; Paludo et al., 2009).

The TCTP (translationally-controled tumor protein) family represents a family of proteins that are highly conserved, ubiquitously

☆ GenBank data deposition information for *L. intermedia* TCTP cloned cDNA is: GenBank ID: JN544929.

* Corresponding author at: Department of Cell Biology, Federal University of Paraná, Jardim das Américas, 81531-990 Curitiba, Paraná, Brazil.
Fax: +55 41 3266 2042.
E-mail address: veigass@ufpr.br (S.S. Veiga).

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expressed in eukaryotes and widely expressed in various tissues and cell types. The name TCTP is based on the first protein of this family identified, which originated from a human mammary carcinoma and on the observation that TCTP is regulated at the translational level and is also called HRF (histamine-releasing factor) (Gachet et al., 1999; Bommer and Thiele, 2004).

Herein, we describe the cloning, expression and purification of a novel, translationally-controlled tumor protein (LiTCTP) family member from the cDNA library of the *Loxosceles intermedia* venom gland. We named the recombinant molecule LiRecTCTP and evaluated the biological activity of this toxin on vascular permeability. We also assessed its relationship to mammalian TCTPs from normal and tumor cells. The results described herein identified this LiTCTP as a toxin from *L. intermedia* venom and one of the molecules responsible for the histamine-associated symptoms in loxoscelism.

2. Materials and methods

2.1. Reagents

The whole venom from *L. intermedia* was obtained by electrostimulation (15 V) of the cephalotorax of spiders, solubilized in PBS and maintained frozen until use (Feitosa et al., 1998). IgGs were purified from serum using a mixture of Protein-A and Protein-G sepharose beads (Amersham Biosciences, Piscataway, USA). Ni^{2+}-NTA agarose was purchased from Invitrogen (Carlsbad, USA), and DEAE agarose Bio-Gel was from Bio-Rad (Hercules, USA). F12, RPMI and DMEM media were purchased from Cultilab (Campinas, Brazil). Evans Blue dye was purchased from Vetec (São Paulo, Brazil).

2.2. cDNA cloning

cDNA for *L. intermedia* TCTP was isolated from a previously built venom gland library as described (Gremski et al., 2010; Chaim et al., 2006). Briefly, randomly chosen colonies were transferred to 1.5 mL microtubes containing 20 μL water, and the suspensions were incubated in a boiling water bath for 10 min. The tubes were centrifuged for 30 min at 20,000 × *g* (4 °C), and sequencing reactions were performed on both strands of DNA from the supernatants using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI3500 Genetic Analyzer (Applied Biosystems, Warrington, UK). The putative protein product based on the sequenced cDNA was used to search NCBI's GenBank protein databases (Altschul et al., 1997). TCTP sequences from 13 different organisms were retrieved from GenBank and aligned with the cDNA sequence of the *L. intermedia* TCTP using the software CLUSTAL X2 in the multiple alignment mode (Thompson et al., 1994). The resulting alignment was used to build a phylogenetic tree using the software MEGA 4.0 with the maximum parsimony method, and the consensus tree was inferred from a 1000 bootstrap replicate test. The tree was rooted with the sequence from *Giardia lamblia* (accession number XP_768559) as it is accepted as the closest organism to an ancestral eukaryote (Hinojosa-Moya et al., 2008).

2.3. Recombinant protein expression

The *L. intermedia* TCTP cDNA was amplified by PCR using the following gene-specific primers: forward primer, 5′-CCGCTCGAG**ATG**ATCATTTTTAAGGATTTATTAAC-3′ (the *Xho*I restriction site is underlined, and the initiation codon is in bold); reverse primer, 5′-CGGGATCC**TTA**AACTTTTTCTTCGTCCAAAC-3′ (the *Bam*HI restriction site is underlined, and the termination codon in bold). The amplified product was digested with *Xho*I and *Bam*HI enzymes (Fermentas, Hanover, MD, USA) and cloned into the pET-14b vector (Novagen, Madison, USA). The correct construction was confirmed by DNA sequencing. The pET-14b cDNA construct was transformed into one-shot *E. coli* BL21(DE3) pLysS competent cells (Invitrogen, Carlsbad, USA) and plated on LB agar medium containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). One colony was then incubated in 10 mL of LB broth with the same antibiotics at the concentrations described above and allowed to grow overnight at 37 °C. This culture was inoculated into 1 L of LB broth with antibiotics and grown at 37 °C until the OD at 550 nm reached 0.5. IPTG (isopropyl β-d-thiogalactoside) was added at a final concentration of 0.1 mM, and induction of the culture was performed for 4 h at 23 °C.

2.4. Protein purification

The sequence cloned into the pET-14b vector was expressed in the One-Shot *Escherichia coli* BL21 (DE3) pLysS cells as a fusion protein with a 6× His-tag at the N-terminus was purified by affinity chromatography using Ni^{+2}-NTA. *E. coli* cells were lysed by thaw–freeze cycles and disrupted by 6 cycles of sonication. The lysed materials were centrifuged (20,000 × *g*, 20 min, 4 °C), and the supernatants were incubated with 1 ml Ni^{2+}-NTA beads for 1 h at 4 °C. The recombinant protein was eluted with 10 ml of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 250 mM imidazole), and 1 ml fractions were collected and analyzed by 12.5% SDS-PAGE under reducing conditions. Fractions were pooled and dialyzed against Bis–Tris buffer (Bis–Tris 20 mM, NaCl 10 mM, pH 7.2). This protein fraction was then submitted to ion-exchange chromatography using DEAE agarose (pH 7.2, NaCl 10 mM). Elution was achieved by performing a stepwise gradient with increasing NaCl concentrations ranging from 0.01 to 1.5 M and the peak of elution was at 25 mM.

2.5. Animals

Adult Swiss mice (25–30 g) and adult rabbits weighing approximately 3 kg from the Central Animal House of the Federal University of Parana were used for *in vivo* experiments with whole venom and recombinant toxin. All procedures involving animals were carried out in accordance with "Brazilian Federal Laws", following the ethical subcommittee on research animal care agreement number 340 of the Federal University of Parana.

2.6. Antibodies

Polyclonal antibodies to whole venom were previously obtained as described (Luciano et al., 2004). Likewise, hyperimmune sera against LiRecTCTP were produced in a rabbit using purified LiRecTCTP as an antigen and complete Freund's adjuvant (Sigma) for the first primary injection at one subcutaneous (SC) point and two intramuscular (IM) points (20 μg of protein divided into three parts was used as the total antigen). As a booster for the second, third, and fourth injections, incomplete Freund's adjuvant (Sigma) was similarly injected at 3-week intervals. Twelve days after the last immunization, blood was collected and the production of specific antibodies analyzed by ELISA and Western blotting (Harlow and Lane, 1988).

2.7. Cell culture

HL-60 and B16-F10 cell lines were obtained from the American Type Tissue Culture Collection (Manassas, USA). Rabbit aorta endothelial cells, RAEC, were obtained courtesy of Professor Helena Bonciani Nader (the Federal University of São Paulo – School of Medicine, São Paulo, Brazil) and were grown in F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Cultilab, Campinas, Brazil). HL-60 (a promyeloblast cell line) and B16-F10

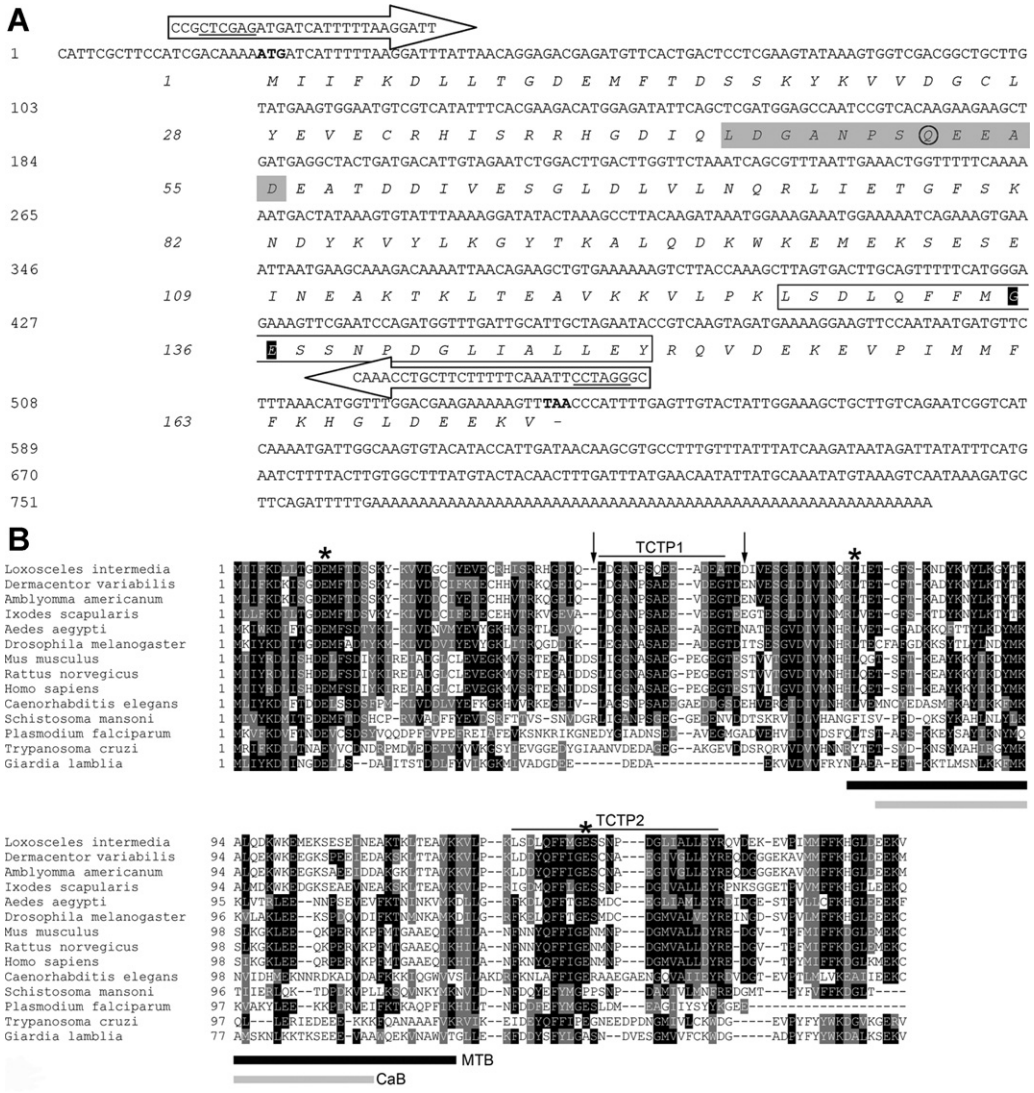

Fig. 1. Molecular cloning of LiTCTP: a novel TCTP-related toxin from *Loxosceles intermedia*. (A) The cDNA and the deduced amino acid sequences of LiTCTP. Arrows show the annealing position of primers containing restriction sites for endonuclease *Xho* I (sense primer) and *Bam* HI (antisense primer). Start and stop codons are in bold. For the protein sequence, the TCTP2 signature is highlighted in the black box and the TCTP1 signature is shaded in gray. Glutamine (circled) is not conserved among other organisms, which does not allow the ProSite Database to identify this signature in LiTCTP. (B) Multiple alignment of TCTP protein sequences from different species. Sequence alignment was performed using the Clustal X2 program and formatted with the BOXSHADE 3.2 program. Fully conserved positions are shaded in black and conservative substitutions are in gray. Asterisks indicate crucial amino acids for the interaction of TCTPs with monomeric GTPases. Serines phosphorylated by the Plk enzyme are indicated by arrows. The two lines above the sequences indicate TCTP signatures 1 and 2. The microtubule binding site (MTB) and calcium binding site (CaB) are overlapping and represented in the figure by black and gray bars, respectively.

(murine melanoma) cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Cultilab) supplemented with 5% FCS (LGC Biotecnologia, Cotia, Brazil). All media were supplemented with 40 mg/L gentamycin (Schering-Plough, Kenilworth, USA). The cell cultures were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.8. Gel electrophoresis and immunoblotting

The protein concentrations of different samples and LiRecTCTP were determined by the Coomassie Blue method (BioRad, Hercules, USA) (Bradford, 1976). For protein analysis, 12.5% SDS-PAGE was performed under reduced conditions, and for immunoblotting, proteins were transferred onto nitrocellulose filters and immunostained with hyperimmune rabbit serum (1:2000), which reacts with LiRecTCTP, or purified IgG against whole venom toxins (25 μg/mL) (Harlow and Lane, 1988). The molecular mass markers were acquired from Sigma. Alternatively, for TCTP immunodetection in tumor cell extracts, protein extracts from the HL-60, B16-F10 and RAEC cell lines (30 μg) were run under reducing conditions in a 12.5% SDS-PAGE, transferred onto nitrocellulose filters and immunostained using rabbit pre-immune and hyperimmune serum against LiRecTCTP (1:5000).

2.9. Paw edema forming activity

Paw edema development was measured at different time intervals (Ribeiro et al., 2007; Gremski et al., 2007). Briefly, groups of five mice were injected subcutaneously into the right hind paw with whole venom (10 μg) or LiRecTCTP (20 μg dissolved in 36 μL sterile PBS). Control mice were injected with 36 μL PBS or 20 μg of recombinant GFP, as a non-related recombinant protein sample (for negative controls). Identically, mice were injected with increasing concentrations of LiRecTCTP (1, 10, and 20 μg dissolved in 36 μL sterile PBS). Edema was

evaluated by examining changes in paw thickness using a calibrated digital micrometer (Digimess, Sao Paulo, Brazil) at the following time points: immediately after SC injection (t zero), and 5, 10, 20, 30, 60, 120, 240, 360 and 720 min after injection.

2.10. Effects on vascular permeability

Changes in vascular permeability were assessed by visualizing extravasation of Evans Blue dye into the extravascular compartment of the skin (Appel et al., 2008; Udaka et al., 1970). A dilution of the dye in PBS solution (30 mg/kg of mice) was injected intravenously prior to intradermal injection of samples (10 μg) of LiRecTCTP, recombinant GFP (negative control) and crude venom ($n = 3$ per treatment). PBS was used as a vehicle control. After 30 min, animal were euthanized and dorsal skin was removed for visualization of dye leakage.

2.11. Statistical analysis

Paw edema measurement results are presented as the mean ± S.E.M., and the statistical analyses were performed using analysis of variance (ANOVA) with the post hoc Tukey test in the GraphPad InStat program version 3.10 for Windows. Results were considered significant when $p \leq 0.01$.

3. Results

3.1. Molecular cloning and multiple alignment analysis of TCTP homologous toxin from the L. intermedia venom gland

By screening clones of a cDNA library of the *L. intermedia* venom gland, a cDNA homologous with the translationally-controlled tumor protein (TCTP) was obtained. The putative protein product from this cDNA was designated LiTCTP (from *L. intermedia* TCTP). The complete cDNA sequence of LiTCTP comprises 536 bp and has an open reading frame that encodes a deduced 172-amino acid protein. The calculated molecular mass of the mature protein for LiTCTP was 22.3 kDa and the pI 4.7, as predicted (Protparam tool from ExPASy) (Gasteiger et al., 2003) (Fig. 1A). A protein-BLAST search using GenBank database revealed that LiTCTP is homologous to other TCTP proteins from a variety of species. This homology was investigated by a multiple sequence analysis of the putative protein products of LiTCTP. LiTCTP is most homologous to TCTP from the ixodid ticks *Ixodes scapularis* and *Amblyomma americanum* sharing 69% of sequence identity, as well the tick *Dermacentor variabilis* with respectively 68% of identity (Fig. 1B).

3.2. The phylogenetic relationship of LiTCTP with other TCTP family members

A phylogenetic tree (Fig. 2) was rooted using the *Giardia lamblia* TCTP sequence because some authors have suggested that this parasitic protist is the closest to a eukaryotic ancestor, as indicated by its basal evolutionary position based on analysis of the *G. lamblia* genome (Hinojosa-Moya et al., 2008; Morrison et al., 2007). LiTCTP forms a cluster with TCTPs from ticks that is supported by a high bootstrap value. The TCTP protein from *Dermacentor variabilis* has been described as a functional histamine-releasing factor and is biologically related to TCTPs from others ticks (Mulenga and Azad, 2005; Mulenga et al., 2003). This analysis demonstrates that the *Loxosceles intermedia* TCTP protein is closely related to histamine-releasing factors of ixodid ticks.

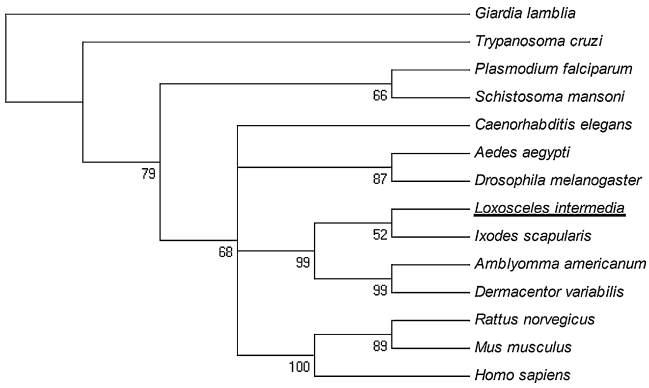

Fig. 2. Phylogenetic relationship of LiTCTP to others TCTP family members. A phylogenetic tree was generated with the program MEGA 4.0, based on the alignment made with Clustal X2. Only branches with bootstrap support greater than 50% are shown. The phylogeny of TCTP proteins reflects that of eukaryotes and suggests an early origin for the protein. LiTCTP is grouped with the TCTP proteins from ticks, which were described as histamine-releasing factors.

3.3. Expression and purification of LiRecTCTP

Expression experiments were performed in *E. coli* BL21(DE3)pLysS cells. The expression of the recombinant toxin was optimal when induced for 3.5 h with 0.1 mM of IPTG. Recombinant toxins were purified from the soluble fraction of cell lysates submitted to 2 steps of chromatography, Ni-NTA agarose and DEAE-sepharose. Recombinant toxin was eluted from IMAC Ni-chromatography (immobilized metal–ion affinity chromatography) resulting in a fraction enriched in LiRecTCTP. The protein fraction was then submitted to ion exchange chromatography using DEAE agarose (pH 7.2, NaCl 10 mM) and was eluted with a high degree of purity using 25 mM of NaCl (Fig. 3, lane 7).

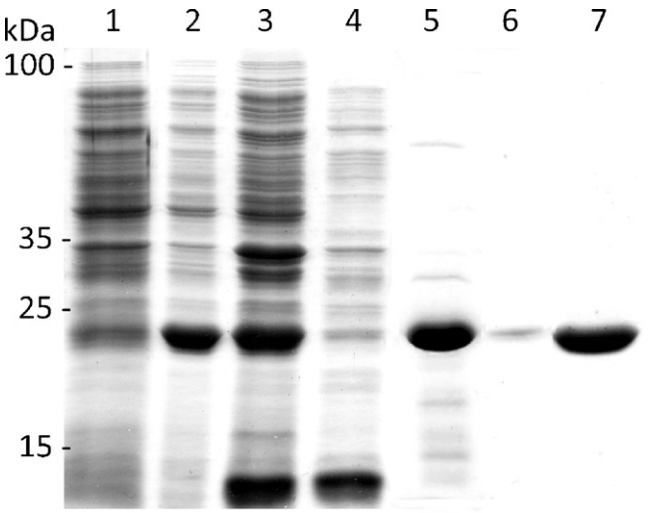

Fig. 3. Expression and purification of recombinant LiTCTP (LiRecTCTP). SDS-PAGE (12.5%) analysis of recombinant TCTP protein expression stained with Coomassie blue dye. Lanes 1 and 2 show *E. coli* BL21(DE3)pLysS cells resuspended in SDS-PAGE gel loading buffer before and after induction for 3.5 h with 0.1 mM IPTG, respectively. Lanes 3 and 4 depict the supernatant from cell lysates obtained by freezing and thawing in extraction buffer before and after incubation with Ni^{2+}-NTA beads, respectively. Lane 5 shows eluted recombinant protein from Ni^{2+}-NTA beads. Lane 6 shows the void from DEAE agarose chromatography and lane 7 shows LiTCTP protein after elution by DEAE chromatography. Molecular mass markers are shown on the left.

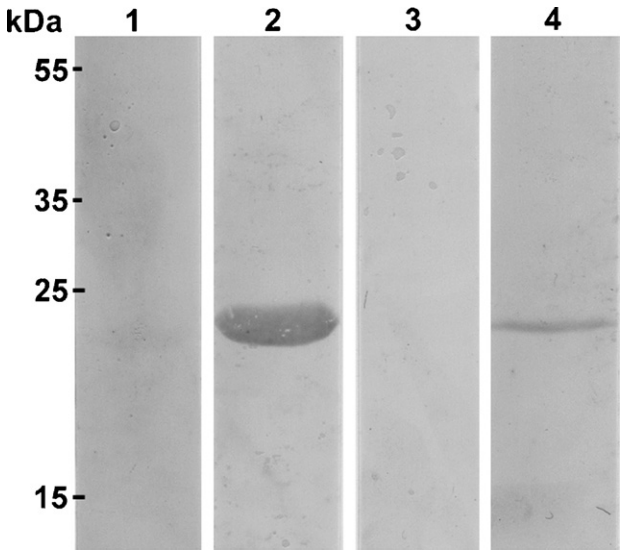

Fig. 4. LiTCTP immunoblotting. Proteins were separated by SDS-PAGE (12.5%) and transferred onto nitrocellulose membranes. Lanes 1 and 2 contain 5 μg of *L. intermedia* recombinant TCTP exposed to 25 μg/mL of rabbit preimmune IgG (1) and hyperimmune IgG against *L. intermedia* venom (2). Lanes 3 and 4 contain 40 μg of *L. intermedia* whole venom exposed to preimmune rabbit antisera (3) and hyperimmune antisera against recombinant LiTCTP (4), both diluted 1:2000. Alkaline-phosphatase-conjugated goat anti-rabbit IgG was used as a secondary antibody and the reaction was developed with BCIP/NBT. Molecular mass markers are shown on the left.

3.4. Immunological cross-reactivity of LiRecTCTP and native venom toxins

Immunoblot analysis (Fig. 4) with polyclonal antibodies produced against LiRecTCTP in rabbits and with polyclonal antibodies against whole venom toxins from *L. intermedia* established a relationship between LiTCTP and brown spider venom. The cross-immunoreactivity of antisera for both LiRecTCTP and native LiTCTP present in the venom demonstrated that whole venom contains a protein similar to this recombinant toxin that has the same immunogenic epitopes.

3.5. Paw edema-forming activity of LiRecTCTP

The edema that develops following an exacerbated inflammation reaction to a brown spider bite is very well described for whole venom and in response to recombinant phospholipase-D (Ribeiro et al., 2007; Paludo et al., 2009). However, recombinant isoforms of phospholipase-D were not able to induce paw edemas of the same intensity as observed in whole venom tests. To demonstrate the edematogenic effect of LiTCTP, we compared the abilities of whole venom, LiRecTCTP, GFP (green fluorescent recombinant protein) and PBS to induce subcutaneous edema *in vivo* in a mouse model. GFP was used as a negative control for purified recombinant molecules and PBS as a control for the vehicle used for administration. As shown in Fig. 5, we observed that LiRecTCTP evoked edema in a time- and concentration-dependent manner (especially, between 1 and 10 μg, and an edema saturation tendency for 20 μg under the experimental conditions used), whereas GFP displayed only residual activity. The subcutaneous injection of brown spider venom and LiRecTCTP (10 μg/paw for whole venom and 20 μg/paw for LiRecTCTP) triggered an edema formation time curve (0–720 min) which started increasing around 5 min (Fig. 5A). The highest peak of edema formation was observed at 5 min for LiRecTCTP. The LiRecTCTP edematogenic effect started rapidly decreasing until a minimum thickness was reached after 240 min.

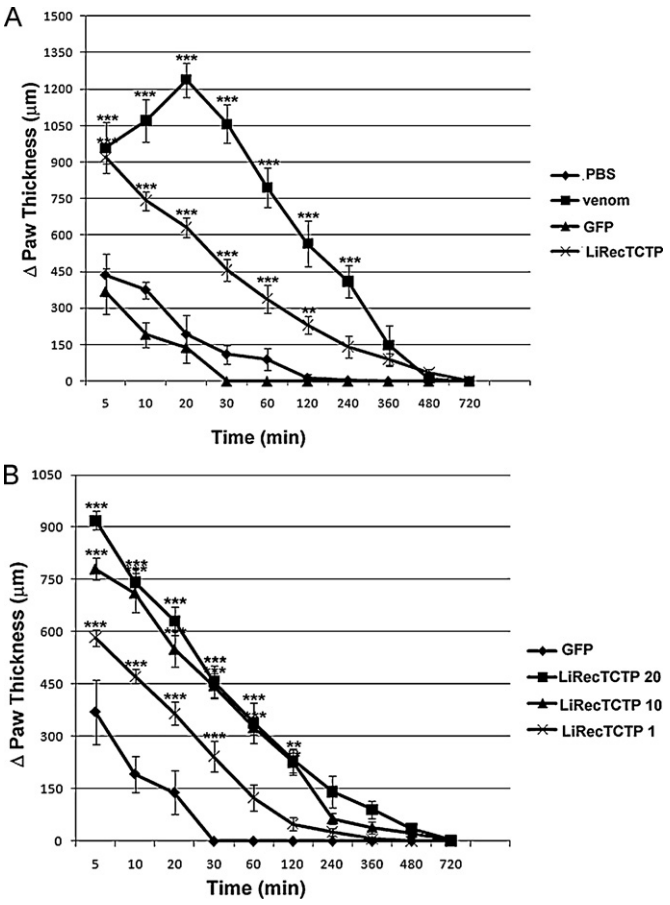

Fig. 5. Paw edema induced by subcutaneous injections of LiRecTCTP. (A) LiRecTCTP and whole venom elicited mouse paw edemas in a time dependent manner beginning at 5 min after injection (LiRecTCTP-induced maximum edema), and absent after 240 min. Values represent the differences between the edema after injection with LiRecTCTP, whole venom, GFP (negative control of a purified recombinant molecule) or PBS (vehicle control) and initial thickness before injections (basal thickness). For LiRecTCTP and whole venom, all points up to 120 min are significantly different from vehicle (PBS) values. Edema induced by LiRecTCTP followed the pattern observed with whole venom injection and regressed to basal thickness 240 min after the injection for LiRecTCTP and after 360 min for whole venom. (B) LiRecTCTP induced a mouse paw edema in a concentration dependent way. Each point represents the mean ± S.E.M for five animals. Significantly different from control values: $^{***}p \leq 0.001$ and $^{**}p \leq 0.01$ (Student's unpaired *t*-test).

LiRecTCTP affected edema measurements in a dose-dependent manner (1, 10 and 20 μg/paw) (Fig. 5B).

3.6. Effect on vascular permeability

Additionally, we tested whether LiRecTCTP could cause changes in vascular permeability by observing vascular leakage of Evans Blue dye in mice injected with whole venom and LiRecTCTP. Recombinant GFP and PBS were used as a negative and vehicle control, respectively. Injection of crude venom caused an intense dye leakage localized around the injection point. Injection of LiRecTCTP also resulted in vascular permeability, but mice presented a more diffuse pattern of dye leakage with several extravasation points near the injection site. Recombinant GFP and PBS injected mice showed no alterations in vascular permeability (Fig. 6).

3.7. Immunological cross-reactivity of polyclonal serum against LiRecTCTP and tumor cell line extracts

To further evaluate the presence of LiTCTP homologous proteins in different cell types and strengthen the idea of a conserved family

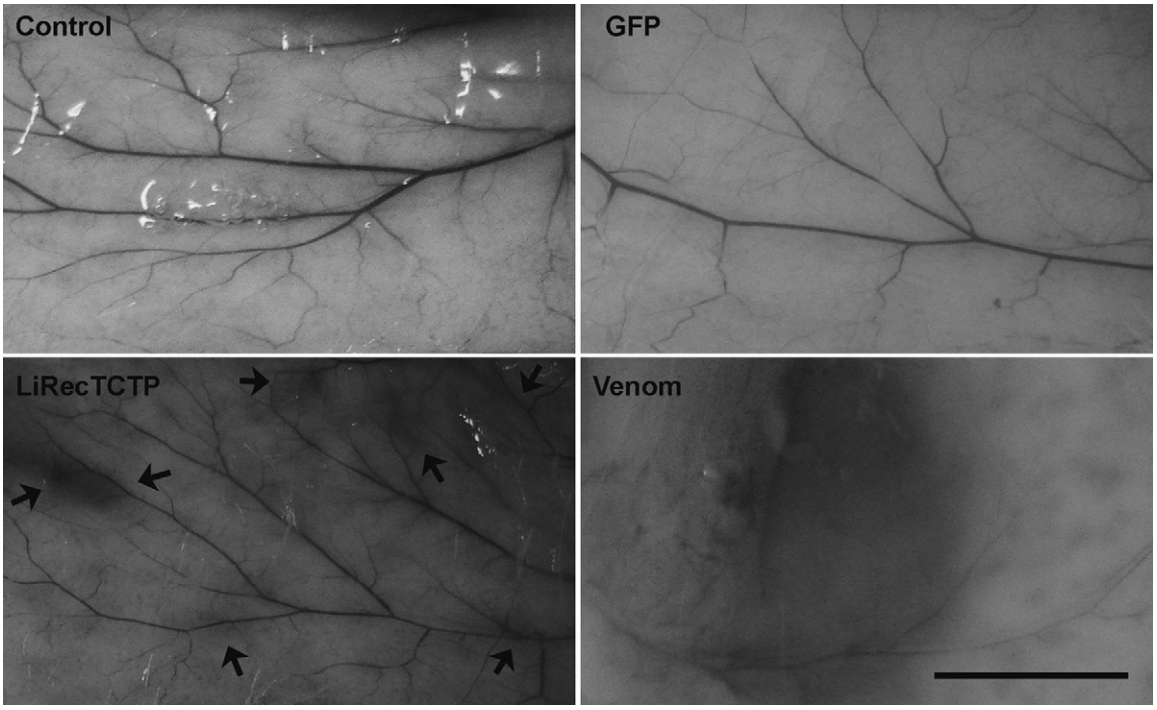

Fig. 6. Effect of LiRecTCTP on vascular permeability of skin vessels. Mice were injected intradermally with 10 μg of LiRecTCTP, crude venom and recombinant GFP (negative control). PBS was used as a vehicle control. Dye leakage was induced by crude venom and LiRecTCTP compared to minimal changes in vascular permeability in animals treated with GFP and PBS. While venom evoked a localized and intense dye extravasation, LiRecTCTP induced several points of vessel permeability suggesting a systemic response. Scale bar points 1 cm.

of molecules in different organisms, we performed western blotting analysis using cell extracts and the antibody raised against LiRecTCTP. This evaluation included cell extracts from tumor cell lines, such as the murine highly metastasizing melanoma cell line B16-F10 and the human promyelocytic leukemia cell line HL-60, and extracts from the rabbit aortic endothelial cell line RAEC. As depicted in Fig. 7, lanes 2, 4, and 6 containing B16-F10, HL-60 and RAEC cell extracts, respectively, reacted positively to hyperimmune serum against LiRecTCTP at different mass ranges for the proteins in nitrocellulose membranes. As a negative control, preimmune serum did not reveal any unspecific reactions when exposed to the same cell extracts (lanes 1, 3, and 5).

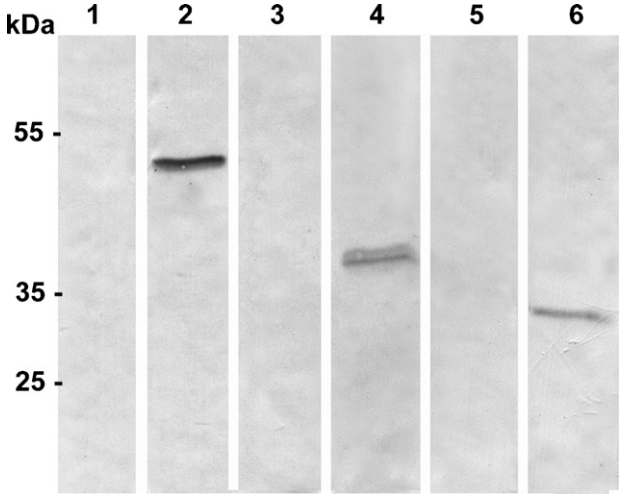

Fig. 7. LiRecTCTP-specific antibodies recognize proteins in extracts from tumor and non-tumor cell lines. B16–F10 (1–2), HL-60 (3–4) and RAEC (5–6) cell extracts (30 μg) were separated by SDS-PAGE (12.5%) and transferred onto nitrocellulose membranes. LiTCTP related proteins were identified using antisera hyperimmune (2, 4, and 6) and preimmune against LiRecTCTP (1, 3, and 5), both diluted 1:5000. Alkaline-phosphatase conjugated goat anti-rabbit IgG was used as a secondary antibody, and the reaction was developed with BCIP/NBT. Molecular mass markers are shown on the left.

4. Discussion

The TCTP family has attracted much attention for its crucial role in many cellular functions such as cell division and proliferation and more recently for its extracellular activity as a histamine-releasing factor (Bommer and Thiele, 2004; Bheekha-Escura et al., 2000). In 1990s, an IgE-dependent histamine-releasing factor (HRF) with sequence homology to TCTP from mice and humans was identified and cloned (named p21 and p23, respectively) (MacDonald et al., 1995). Later, it was demonstrated that this HRF could promote the release of histamine from basophiles independent of IgE, and evidences suggested an interaction of this HRF with a unique receptor on the cell surface (Bheekha-Escura et al., 2000; Kang et al., 2001; Kim et al., 2009).

Sequence analysis of LiTCTP showed that it contains the signature region of high sequence homology termed TCTP-2 and also the TCTP-1 region, which is present but with a non-conserved substitution (glutamine instead of alanine at position 51) (Fig. 1A). This substitution was suggested to represent a minor change in LiTCTP structure, which would not affect the Cβ-strand because it occurs in a flexible region of the molecule and the bigger lateral chain of glutamine would be admitted with no representative structural alterations. Within the TCTP-2 signature region of LiTCTP, the calcium and microtubule binding sites are total or partially conserved, and amino acids related to GTPases interactions are also conserved (Fig. 1A and B).

Among species of the same genus, TCTPs are completely conserved (Bommer and Thiele, 2004). Multiple sequence alignment analysis and phylogenic trees support the high degree of identity of LiTCTP with ixodid tick TCTPs (Figs. 1B and 2). TCTP from

Demacentor variabilis was characterized as a histamine-releasing factor and it was shown to biologically related with TCTP proteins from other ticks (Mulenga et al., 2003). The overall identity of LiTCTP is about 69% with TCTPs from *Ixodes scapularis*. Ixodid ticks such as *Ixodes scapularis, Dermacentor variabilis* and *Amblyomma americanum* are also arthropods from the arachnida class that includes *Loxosceles intermedia*.

The *L. intermedia* venom gland transcriptome revealed that TCTP is represented in the venom, corresponding to 0.4% of the transcripts that encode toxins (Gremski et al., 2010). In the case of the *Loxosceles* venom gland, TCTP and other constituents of whole venom are secreted via holocrine secretion as revealed by ultrastructural studies of the venom gland (Gremski et al., 2010; dos Santos et al., 2000). The LiTCTP sequence obtained does not present a signal peptide for endoplasmic reticulum (ER) translocation (Fig. 1A) suggesting that it is secreted by the exosomal, non-classical pathway. TCTP secretion from cells proceeds via an ER/Golgi-independent pathway, probably mediated by secreted vesicles called exosomes, which has been suggested as a possible pathway for non-classical protein secretion (Amzallag et al., 2004; Hinojosa-Moya et al., 2008). The TCTP protein has been found in biological fluid of asthmatic or parasitized patients, in saliva from ticks (Hinojosa-Moya et al., 2008; Bommer and Thiele, 2004) and in the crude venom of *L. intermedia* as described herein (Fig. 4).

It has already been shown that *L. intermedia* venom increases vascular permeability and induces vascular relaxation in rats (Rattmann et al., 2008), and that these effects occur due to the ability of venom to degranulate mast cells and release mediators such as histamine (Paludo et al., 2009). LiTCTP seems to be an earlier and quicker component of edema formation compared with the inflammatory response in mouse paws triggered by whole venom (Fig. 5). LiRecTCTP also induced microvascular permeability of skin vessels (Fig. 6). The extravasation pattern was different from that induced by crude venom, presenting several leakage points and a less localized response. The presence of histamine in the envenomation site can cause pain, edema, erythema and endothelial changes such as increased vascular permeability and vasodilation, all of which contribute to the systemic dispersion of venom components. Moreover, inflammatory responses can be related to both mast cells and histamine (Paludo et al., 2009; Mekori and Metcalfe, 2000; Weisel-Eichler and Libersat, 2004).

The TCTP family possesses a wide range of biological functions (Choi and Hsu, 2007; Bommer and Thiele, 2004). Although TCTPs are involved in various biological events, the primary physiological roles for these proteins are still unknown (Sun et al., 2008).

Different studies have identified several proteins that interact with TCTP linking this protein to diverse signaling pathways. Interactions between TCTP and the following molecules have already been observed: tubulin (Gachet et al., 1999; Rinnerthaler et al., 2006; Bazile et al., 2009), actin-F (Bazile et al., 2009), the mammalian Plk (Yarm, 2002), translation elongation factors eEF1A and eEF1Bbeta (Cans et al., 2003), Mcl-1 (Liu et al., 2005; Li et al., 2001), TSAP6 (Amzallag et al., 2004), Na,K-ATPase (Jung et al., 2004), Bcl-XL (Yang et al., 2005) and Chrf (Burgess et al., 2008). TCTP is involved in cell cycle regulation and proliferation, malignant transformation and in the protection of cells against various stress conditions and apoptosis (Bommer and Thiele, 2004; Chen et al., 2007; Susini et al., 2008). Moreover, the protein has calcium-binding activity and is capable of stabilizing microtubules, a property that may be related to participation of TCTP in cell cycle control, as it TCTP has been to interact with a checkpoint protein (Chrf) (Burgess et al., 2008). There is also evidence that TCTP may be a functional link between microtubules and mitochondria that participates in the correct localization of mitochondria during cell division (Rinnerthaler et al., 2006).

As seen in Fig. 1B, Plk phosphorylation sites were not observed in LiTCTP, they are only conserved in human, mouse and rat TCTP proteins, which are evolutionarily close to each other. Moreover, none of the LiTCTP serines are Plk phosphorylation sites [E–G–(A/E)–(I/G)–(D/T)–(D/E)–S–(L/T)–(I/V)]. As TCTP phosphorylation at these sites triggers its disassociation from microtubules allowing mitosis progression specifically from metaphase to anaphase (Bommer and Thiele, 2004), their absence in LiTCTP suggests that for brown spiders, and other organisms, this toxin is probably not involved in microtubule stabilization during the cell cycle (Yarm, 2002).

Down-regulation of TCTPs has been implicated in biological models of tumor reversion (Tuynder et al., 2002, 2004), and the protein is now the target of various anticancer drugs (Telerman and Amson, 2009; Efferth, 2005). TCTP protein levels are up-regulated in cancer cells and in human tumors (Telerman and Amson, 2009; Efferth, 2005; Slaby et al., 2009; Ma et al., 2009). TCTP has been proposed as a potential cancer biomarker (van de Sande et al., 2006; Slaby et al., 2009; Kim et al., 2008) and therapeutic target (Zhu et al., 2008). Drugs that inhibit TCTP activity resulted in tumor growth inhibition both *in vitro* and *in vivo* (Tuynder et al., 2004). Telerman and Amson (2009) reported that these drugs disrupt the interaction of TCTP with partner proteins that bind directly to TCTP and increase its secretion from the cell thereby lowering intracellular concentrations of TCTP. Antibodies raised against the TCTP from *L. intermedia* allowed for the immunodetection of mammalian native TCTP molecules in the tumor cell lines studied herein (B16-F10 and HL-60, Fig. 7). This raises another promising possibility for this recombinant toxin in the experimental oncology field along with its potential uses in the immunological and allergenic biomedical areas. Knowledge about LiTCTP can be used to elucidate not only different aspects of loxoscelism biology, but can also provide new insights into the diverse functions of this family of proteins and the interrelation between them.

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