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Brown Spider Phospholipase-D Containing a Conservative Mutation (D233E) in the Catalytic Site: Identification and Functional Characterization

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ABSTRACT

Brown spider (*Loxosceles* genus) bites have been reported worldwide. The venom contains a complex composition of several toxins, including phospholipases-D. Native or recombinant phospholipase-D toxins induce cutaneous and systemic loxoscelism, particularly necrotic lesions, inflammatory response, renal failure, and hematological disturbances. Herein, we describe the cloning, heterologous expression and purification of a novel phospholipase-D toxin, LiRecDT7 in reference to six other previously described in phospholipase-D toxin family. The complete cDNA sequence of this novel brown spider phospholipase-D isoform was obtained and the calculated molecular mass of the predicted mature protein is 34.4 kDa. Similarity analyses revealed that LiRecDT7 is homologous to the other dermonecrotic toxin family members particularly to LiRecDT6, sharing 71% sequence identity. LiRecDT7 possesses the conserved amino acid residues involved in catalysis except for a conservative mutation (D233E) in the catalytic site. Purified LiRecDT7 was detected as a soluble 36 kDa protein using anti-whole venom and anti-LiRecDT1 sera, indicating immunological cross-reactivity and evidencing sequence-epitopes identities similar to those of other phospholipase-D family members. Also, LiRecDT7 exhibits sphingomyelinase activity in a concentration dependent-manner and induces experimental skin lesions with swelling, erythema and dermonecrosis. In addition, LiRecDT7 induced a massive inflammatory response in rabbit skin dermis, which is a hallmark of brown spider venom phospholipase-D toxins. Moreover, LiRecDT7 induced in vitro hemolysis in human erythrocytes and increased blood vessel permeability. These features suggest that this novel member of the brown spider venom phospholipase-D family, which naturally contains a mutation (D233E) in the catalytic site, could be useful for future structural and functional studies concerning loxoscelism and lipid biochemistry. Highlights: 1- Novel brown spider phospholipase-D recombinant toxin contains a conservative mutation (D233E) on the catalytic site. 2-LiRecDT7 shares high identity level with isoforms of *Loxosceles* genus. 3-LiRecDT7 is a recombinant protein immunodetected by specific antibodies to native and recombinant phospholipase-D toxins. 4-LiRecDT7 shows sphingomyelinase-D activity in a concentration-dependent manner, but less intense than other isoforms. 5-LiRecDT7 induces dermonecrosis and inflammatory response in rabbit skin. 6-LiRecDT7 increases vascular permeability in mice. 7-LiRecDT7 triggers direct complement-independent hemolysis in erythrocytes. J. Cell. Biochem. 114: 2479–2492, 2013.

KEY WORDS: BROWN SPIDER; VENOM; DERMONECROTIC TOXIN; PHOSPHOLIPASE-D; CLONING; RECOMBINANT PROTEIN

The authors declare no conflict of interest.

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Spiders of the *Loxosceles* genus are commonly known as brown spiders based on their body colors that range from fawn to dark brown. At present, 103 species of spiders from the *Loxosceles* genus have been described and are distributed worldwide [Platnick, 2012]. There are 12 species of *Loxosceles* genus spiders in Brazil [Gonçalves-de-Andrade et al., 2012]. Loxoscelism is the term used to describe the lesions caused from bites by *Loxosceles* genus spiders [da Silva et al., 2004; Appel et al., 2005; Chaim et al., 2011a]. Traditionally, loxoscelism is divided in two clinical conditions: cutaneous and systemic loxoscelism. Cutaneous loxoscelism refers to the typical skin lesion with dermonecrosis, edema, erythema and gravitational spreading that appears after most accidents. Systemic loxoscelism is less common than the cutaneous form, but is responsible for complications following accidents, which might include hematological disturbances, such as thrombocytopenia, disseminated intravascular coagulation, hemolytic anemia, and renal failure [Futrell, 1992; da Silva et al., 2004; Isbister and Fan, 2011].

The volume of venom inoculated by *Loxosceles* genus spiders is typically minute (some microliters) and enriched in proteins of low molecular mass (20–45 kDa). To date, a great number of brown spider venom toxins have been identified in different *Loxosceles* species, including hyaluronidases [Young and Pincus, 2001; Barbaro et al., 2005; da Silveira et al., 2007a; dos Santos et al., 2009], metalloproteases from the astacin family [da Silveira et al., 2007b; Trevisan-Silva et al., 2010], serine proteases [Veiga et al., 2000; Gremski et al., 2010], translationally controlled tumor protein (TCTP) [Sade et al., 2012], insecticidal peptides of the ICK family [Gremski et al., 2010], serine protease inhibitors [Gremski et al., 2010], and members of the phospholipase-D family, also known as dermonecrotic toxins, based on their biological activity [Kalapothakis et al., 2007; Appel et al., 2008; Binford et al., 2009].

Previous studies using native dermonecrotic toxins purified from crude venom have biochemically characterized this toxin as a sphingomyelinase based on its ability to hydrolyze sphingomyelin into choline and ceramide-1-phosphate [Futrell, 1992]. Using recombinant isoforms of dermonecrotic toxins from different *Loxosceles* species, it has recently been postulated that these molecules are phospholipase-D enzymes based on their abilities to hydrolyze other synthetic phospholipids, such as lysophosphatidylcholine, lysophosphatidylinositol, lysobisphosphatidic acid, cyclic phosphatidic acid, and lysoplatelet-activating factor [Lee and Lynch, 2005; Chaim et al., 2011b]. The final release of lipid metabolites such as ceramide-1-phosphate (C1P) from sphingomyelin or lysophosphatidic acid from a lysophospholipid was already described for *Loxosceles* phospholipases-D [van Meeteren et al., 2004; Chalfant and Spiegel, 2005; Lee and Lynch, 2005]. These metabolites are bioactive mediators that play a major role in complex signaling pathways that control several cellular processes and also in various pathophysiologic processes [Hannun, 1994; Anliker and Chun, 2004; Moolenaar et al., 2004]. Thus, these lipid mediators could induce toxicity by activating signaling pathways involved with a variety of pathophysiologic changes [Chaim et al., 2011b].

The phospholipase-D toxins in *Loxosceles* genus spider venoms represent the most biologically and biochemically characterized brown spider venom components and various isoforms of these molecules have previously been reported for different species [Chaim et al., 2011a]. Four biochemically related isoforms of phospholipase-D in a native purified dermonecrotic toxin have been reported in *L. reclusa* venom [Futrell, 1992]. The results from N-terminal sequence studies of *L. boneti* venom have revealed three different isoforms of phospholipase-D in this species [Ramos-Cerrillo et al., 2004]. Similarly, based on proteomic analyses such as two-dimensional electrophoresis, N-terminal amino acid sequencing and mass spectrometry, the presence of at least eleven isoforms of phospholipase-D have been reported for *L. gaucho* venom [Machado et al., 2005]. Six isoforms of phospholipase-D have been identified in *L. intermedia* venom using molecular biology techniques such as cloning, heterologous expression, amino acid alignment and phylogenetic studies [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Appel et al., 2008]. Additional studies have also described multiple members of the phospholipase-D family in other *Loxosceles* venoms, and these isoforms differ in various features, such as their biological activity and substrate specificity [Lee and Lynch, 2005; Kalapothakis et al., 2007; Catalán et al., 2011; Chaim et al., 2011b]. The transcriptome analysis of the venom gland of *L. intermedia* revealed that phospholipases-D represent 20.2% of the total toxin-encoding transcripts present in the gland [Gremski et al., 2010]. An inter-species family of similar phospholipase-D toxins has been postulated, and the noxious effects of *Loxosceles* spp. whole venom are due to the family synergism of these related toxins [Kalapothakis et al., 2007]. Many studies indicate that the gene family of venom phospholipase-D toxins has undergone frequent multiple duplications and occasional functional evolution [Binford et al., 2009].

Here, we describe the cloning, heterologous expression, purification, and functional evaluation of a novel phospholipase-D isoform from a cDNA library of *L. intermedia* venom gland. This novel isoform has conservative the amino acid residues involved in the catalysis and metal ion coordination that are important for phospholipase activity, as previously reported for the other members of the family [Murakami et al., 2006; de Giuseppe et al., 2011], except for a conservative mutation (D233E) in the catalytic site. Taken together, these results strengthen the concept of a gene family encoding different phospholipase-D toxins in the venom of *L. intermedia*. Here, we present evidence of a novel isoform and illustrate the features of a dermonecrotic toxin with a natural mutation in an amino acid residue in the catalytic site.

METHODS

REAGENTS

Whole venom from *L. intermedia* was extracted from spiders captured in the wild with the authorization of the Brazilian Governmental Agency "Instituto Chico Mendes de Conservação da Biodiversidade" number 29801-1, according to [Feitosa et al., 1998]. Polyclonal antibodies to *L. intermedia* crude venom toxins and phospholipase-D "dermonecrotic toxin" (LiRecDT1) were produced in rabbits as previously described [Chaim et al., 2006]. Evans Blue dye was purchased from Vetec (São Paulo, Brazil). NaCl, KCl, $CaCl_2$, Na_2HPO_4, KH_2PO_4, Na_2PO_4, NaH_2PO_4, Imidazole, Lysozyme, and Ágar XLT4 were purchased from Merck (Darmstadt, Germany). Tryptone Type I, Yeast Extract (Himedia, Mumbai, India). Chloramphenicol and

Ampicillin Trihydrate were purchased from USB Corporation (Cleveland, OH). Tris and Sucrose were purchased from Bio-Rad (Hercules, CA) and Sigma–Aldrich (St. Louis, MO), respectively. Xylasine and Ketamine were purchased from Rhobifarma (Hortolândia, São Paulo, Brazil).

cDNA CLONING

The partial cDNA sequence for the dermonecrotic toxin isoform 7 described herein was isolated from a previously constructed venom gland cDNA library [Gremski et al., 2010]. Briefly, venom gland mRNAs were purified from total RNA using a magnetic separation kit, PolyATtract mRNA Isolation System III (Promega Corporation, Madison, WI). A directional *L. intermedia* venom gland cDNA library was constructed using a Creator SMART cDNA Library Construction Kit (BD Biosciences Clontech, Mountain View, CA). The first strand of cDNA was synthesized from purified mRNA, and the second strand was obtained through long distance PCR (LD-PCR), according to the manufacturer's instructions. The cDNA was size fractionated via chromatography to avoid contaminating the library with short length sequences. Competent *Escherichia coli* DH5α cells were transformed with the cDNA library plasmids to amplify the cDNA. The transformants were selected on LB (Luria-Bertani) agar plates containing 34 μg/ml chloramphenicol, and more than 20,000 colonies were obtained.

AMPLIFICATION OF THE 5′ END OF THE cDNA

To obtain the complete 5′ end of phospholipase-D isoform 7 cDNA, a 5′RACE (Rapid Amplification of cDNA Ends) protocol was performed according to Sambrook and Russell [2001], with some modifications. Approximately 1 μg of total RNA was obtained from *L. intermedia* venom glands. The first-strand cDNA was synthesized using the gene-specific reverse primer R1 (5′-CGAACACAACTAGGGT-CAGTTCTG-3′) and Improm-II Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. The cDNA was recovered through ethanol precipitation in the presence of ammonium acetate and was subsequently poly (A)-tailed with terminal deoxynucleotidyl transferase (Fermentas, Hanover, MD) according to the manufacturer's instructions. The modified cDNA was PCR amplified with the (dT) 17-adaptor primer (5′-CGGTACCATG-GATCCTCGAGTTTTTTTTTTTTTTTT-3′) and the nested gene-specific reverse primer R2 (5′-CTTGTGACACCACCTTCTGCAATC-3′) using Pfu DNA polymerase (Fermentas). The PCR product was gel-purified using a PerfectPrep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions and was sequenced on both strands using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on a DNA 3500 Genetic Analyzer automatic sequencer (Applied Biosystems, Warrington, UK). The putative protein product from the sequenced cDNA was used to search the GenBank protein databases at NCBI.

RECOMBINANT PROTEIN EXPRESSION

The cDNAs encoding putative mature phospholipase-D isoform 7 (LiRecDT7) were PCR amplified using primers designed to contain *Nde*I (Fermentas) restriction sites at the 5′ ends (forward primers) and *Bam*HI (Fermentas) sites at the 3′ ends (reverse primers). The PCR products were digested with *Nde*I and *Bam*HI restriction enzymes and subcloned into the pET-14b plasmid (Novagen, Madison, USA) digested with the same enzymes. The recombinant construct was expressed as a fusion protein, with a 6× His-Tag at the N-terminus and a 13 amino acid linker, including a thrombin site between the 6× His-Tag and the mature protein. The expression construct was transformed into *E. coli* SHuffle T7 Express *lysY* cells (New England Biolabs, Ipswich, MA, UK) and plated on LB plates containing 100 μg/ml ampicillin. Single colonies of LiRecDT7 construct were inoculated into LB broth (100 μg/ml ampicillin) and grown overnight at 30°C. This culture was diluted 1:50 into 2 L fresh LB broth/ampicillin and incubated at 30°C until the $OD_{600\,nm} = 0.5$. Recombinant expression was induced with the addition of 0.05 mM IPTG (isopropyl β-D-thiogalactoside; Fermentas) and cells were incubated for 4 h at 30°C. The cells were harvested through centrifugation (4,000*g*, 7 min, 4°C), resuspended in 40 ml of extraction buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole, and 1 mg/ml lysozyme) and frozen overnight at −20°C.

PROTEIN PURIFICATION

The cells were thawed and disrupted through mechanical lysis. The lysed materials were centrifuged (9,000*g*, 30 min, 4°C), and the supernatants were incubated with 500 μl of Ni^{2+}-NTA beads for 1 h at 4°C. The suspensions were loaded onto a column and the packed gel was washed with a buffer containing 50 mM sodium phosphate, pH 8.0, 500 mM NaCl and 30 mM imidazole. The recombinant protein was eluted with 2 ml of elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 250 mM imidazole), and the fractions were collected and analyzed using 12.5% SDS–PAGE under β-mercaptoethanol reducing conditions. The fractions were pooled and dialyzed against phosphate-buffered saline (PBS).

MOLECULAR MODELING OF THE LiRecDT7 AND STRUCTURAL ANALYSIS OF PROTEINS USING BIOINFORMATICS TOOLS

We used the SWISS-MODEL [Kiefer et al., 2009] software (http://swissmodel.expasy.org/) to construct a prediction of the three-dimensional structure of LiRecDT1 and LiRecDT7. The protein used as a model for analysis of the predictions was LiRecDT1 (PDB code: 3RLH), as this toxin has been crystallized and its 3D structure has been solved [de Giuseppe et al., 2011]. The production of the figure presented in the results was produced with the software Open Astex Viewer 3.0 (http://openastexviewer.net/web/), a program for molecular visualization.

SPHINGOMYELINASE-D ACTIVITY ASSAY

The Sphingomyelinase-D activity of purified LiRecDT7 was measured using an Amplex Red Assay Kit (Molecular Probes, Eugene, USA) through the analysis of the sphingomyelinase-D activity of recombinant toxin. In this assay, sphingomyelinase-D activity was monitored using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2 [Chaim et al., 2011b]. First, sphingomyelinase-D hydrolyzes sphingomyelin to yield C1P and choline. Choline is then oxidized to betaine and H_2O_2 through coline oxidase. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with the Amplex reagent at 1:1 stoichiometry to generate the highly fluorescence product, resorufin. LiRecDT1 (positive control) or recombinant LiRecDT7 (5, 10, and 20 μg, in

five trials) were added to the Amplex Red reagent mixture. The reaction tubes were incubated at 37°C for 30 min, and the fluorescence was measured in a microplate fluorimeter (Tecan Infinite® M200, Männedorf, Switzerland) using excitation at 540 nm with emission detection at 570 nm.

DETERMINATION OF HEMOLYTIC ACTIVITY

The hemolysis assay was performed as previously described [Chaim et al., 2011b; Chaves-Moreira et al., 2011]. Washed red blood cells (10^8 cells) were added to each Eppendorf tube containing the appropriate concentrations of LiRecDT7 (0.025, 0.25, 2.5, and 25 μg/ml) in Tris buffer sucrose (TBS; 250 mM sucrose, 10 mM Tris/HCl, pH 7.4 and 280 mOsm\kg H_2O) containing 1 mM $CaCl_2$. For this assay, the experiments were performed in pentaplicate along with negative (in presence of the appropriate amount of TBS with 1 mM $CaCl_2$) and positive (red blood cells in 0.1% v/v Triton X-100) controls. After 24 h of incubation with gentle agitation, the controls and samples were centrifuged (refrigerated Eppendorf microfuge) for 3 min at 1,600 rpm, and the absorbance values for the supernatants were immediately measured at 550 nm (Meridian ELx 800, Auto Reader Diagnostics, Inc., USA). The absorbance values were converted to percent hemolysis using the absorbance values of the positive control as 100% lysis. Blood collection from voluntary students was authorized through agreement of the ethical committee of the Federal University of Paraná.

ANIMALS

Adult Swiss mice (25–30 g) and adult rabbits weighting approximately 3 kg from the Central Animal House of the Federal University of Parana were used for the in vivo experiments with whole venom and recombinant toxins. All procedures involving animals were performed in accordance with "Brazilian Federal Laws," following Ethical Subcommittee on Research Animal Care Agreement number 565 of the Federal University of Parana.

IN VIVO STUDIES ON RABBITS

For the evaluation of the dermonecrotic effect, 10 μg of purified recombinant LiRecDT7 diluted in PBS was injected intradermally into a shaved area of rabbit skin. Dermonecrosis was assessed at 3, 6, and 24 h after injection. For a negative control to assure that bacterium constituent contamination during purification did not influence the results, we used purified recombinant green fluorescent protein (GFP) obtained under the same conditions for LiRecDT7. Rabbits were used in experiments for dermonecrosis because this animal model reproduces skin lesions consistent with those observed in accidents with humans [da Silva et al., 2004]. The experiments were repeated twice for each sample.

HISTOLOGICAL METHODS FOR LIGHT MICROSCOPY

Rabbit skin was collected from animals anesthetized with ketamine and xylazine, and subsequently fixed in "ALFAC" (ethanol absolute 85%, formaldehyde 10%, and glacial acetic acid 5%) for 16 h at room temperature. After fixation, the samples were dehydrated in a graded series of ethanol before paraffin embedding (for 2 h at 58°C). Thin sections (4 μm) were processed for histology. The tissue sections were stained with hematoxylin and eosin (H&E) as previously described [Chaim et al., 2006; da Silveira et al., 2006]. The images were obtained using an Axio Imager Z2 microscope (Carl Zeiss, Jena, DE) equipped with a motorized scanning module VSlider (MetaSystems, Altlussheim, DE).

MEASUREMENT OF VASCULAR PERMEABILITY

The changes in capillary permeability were based on the leakage of plasma protein-bound dye into the extravascular compartment of the skin [Appel et al., 2008]. Evans Blue dye diluted in saline was administered intravenously (30 mg/kg of mice) 5 min prior to sample injections. Whole venom and the recombinant toxins LiRecDT1 and LiRecDT7 (10 μg) were injected intradermally into the dorsal skin of mice ($n = 5$ per treatment). After 30 min, the animals were anesthetized using ketamine and acepromazine, sacrificed and the dorsal skin was removed for the visualization of dye extravasation. For the negative control, animals received only a saline injection without venom toxins. Mice were used because this animal model does not develop dermonecrosis and local hemorrhage following brown spider venom exposure, an event that could mask the interpretation of vascular permeability.

GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Protein content was determined using the Coomassie Blue method (BioRad, Hercules, CA). For protein analysis, 12.5% SDS–PAGE under reduced conditions. For immunoblotting, the proteins were transferred to nitrocellulose filters and immunostained polyclonal with antibodies raised against phospholipase-D isoform 1 (LiRecDT1) or against whole venom toxins. The molecular mass markers were acquired from Sigma.

STATISTICAL ANALYSIS

The statistical analyses were performed using analysis of variance (ANOVA) with a post hoc Tukey test using GraphPad InStat program version 5.00 for Windows XP. Statistical significance was considered when $P \leq 0.05$.

RESULTS

CLONING OF A NOVEL PHOSPHOLIPASE-D ISOFORM FROM *L. intermedia* VENOM GLAND

A partial cDNA encoding for a novel phospholipase-D isoform was obtained through screening clones of a cDNA library of *L. intermedia* venom gland [Gremski et al., 2010]. The complete cDNA sequence was obtained using RT-PCR in a 5′ RACE protocol. The putative protein product from this cDNA was referred to as LiRecDT7 (from *L. intermedia* recombinant dermonecrotic toxin). The other isoforms previously described were the following: LiRecDT1 [Chaim et al., 2006], LiRecDT2 and LiRecDT3 [da Silveira et al., 2006], LiRecDT4 and LiRecDT5 [da Silveira et al., 2007c], and LiRecDT6 [Appel et al., 2008]. The complete cDNA sequence of LiRecDT7 comprises 1,200 bp with a single ORF coding for 300 amino acids with a hydrophobic and putative signal peptide of 18 amino acids (Fig. 1). The calculated molecular mass of the predicted mature protein for LiRecDT7 was 34.4 kDa, with a pI of 5.94.

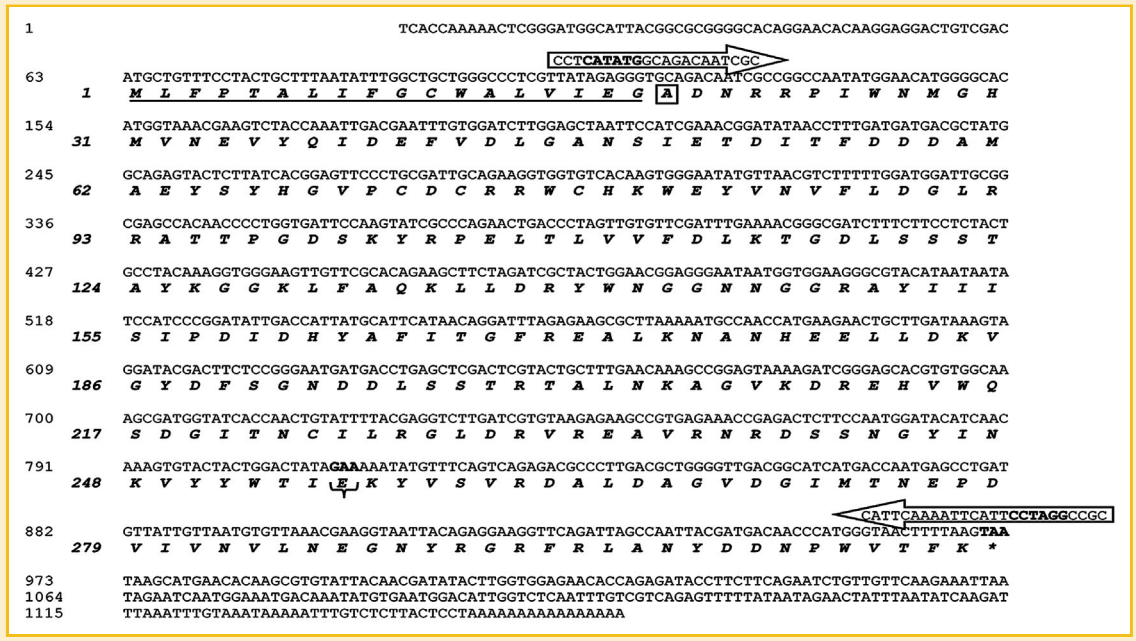

Fig. 1. Molecular cloning of a novel phospholipase-D toxin from *L. intermedia* venom gland cDNA library. Nucleotide and deduced amino acid sequences of cloned phospholipase-D. In the protein sequence, the predicted signal peptide is underlined. The arrows show the annealing positions for the primers used for subcloning into the expression vector, and the restriction sites are highlighted in bold. The alanine in the box indicates the first amino acid of the mature protein with 282 amino acids. The nucleotide and amino acid residue for the conservative substitution for LiRecDT7 (D233E) is highlighted in the bracket. The asterisk corresponds to the stop codon TAA.

MULTIPLE ALIGNMENT ANALYSIS OF THE cDNA-DEDUCED AMINO ACID SEQUENCE AND THE SIMILARITY OF LiRecDT7 WITH OTHER PHOSPHOLIPASE-D TOXIN FAMILY MEMBERS

The BLAST GenBank database search revealed that LiRecDT7 has structural similarity to other LiRecDT family members. The overall identity of LiRecDT7 is approximately 63% with LiRecDT1. From all *L. intermedia* phospholipases-D described, LiRecDT7 is more similar to LiRecDT6, sharing 71% sequence identity. When compared with the other *L. intermedia* dermonecrotic toxin isoforms, LiRecDT7 shows 64% sequence identity to LiRecDT2, 45% identity to LiRecDT3, 60% identity to LiRecDT4, and 46% identity to LiRecDT5 (Fig. 2A). LiRecDT7 also shares 58% identity with LoxTox i5 (EF535254), whose sequence has been previously described [Kalapothakis et al., 2007]. A similarity analysis (Fig. 2B) of these seven phospholipase-D isoform toxins and the cloned cDNAs of other brown spider dermonecrotic toxins revealed that LiRecDT7 was most identical to the *L. hirsuta* toxin (GenBank accession number ACN48948; 91%). These data are consistent with the idea of an intra and inter-species family of brown spider venom phospholipases-D, now containing a novel member.

EXPRESSION, PURIFICATION, AND IMMUNOLOGICAL RELATIONSHIP OF LiRecDT7 TO OTHER BROWN SPIDER MEMBERS

LiRecDT7 was expressed using the N-terminal tag of six histidine residues. Expression experiments were performed in *E. coli* SHuffle T7 Express *lysY* cells. The expression of recombinant protein was optimal when induced for 4 h with 0.05 mM of IPTG. Recombinant phospholipase-D was purified using the soluble fraction of cell lysates under native conditions using Ni^{2+} NTA agarose-chelating chromatography to obtain a 200 μg/L sample of the purified recombinant protein. The SDS–PAGE mobility of the purified recombinant protein, reduced through β-mercaptoethanol treatment, was 36 kDa (Fig. 3A). Differences between the deduced molecular mass and SDS-electrophoretic mobility of LiRecDT7 resulted from the 6× His-tag fusion peptide. Immunoblot analysis using a LiRecDT1 specific antibody and antibodies for whole venom toxins established an immunological relationship between purified recombinant LiRecDT7 and native venom phospholipases-D (dermonecrotic toxins) and LiRecDT1 (Fig. 3B). Both antibodies reacted with purified LiRecDT7, demonstrating that whole venom contains proteins similar to this recombinant toxin, and LiRecDT7 contains similar sequence/epitopes and antigenic cross-reactivity with LiRecDT1, the prototype of this family of toxins.

MOLECULAR MODELING OF LiRecDT7

To model the 3D structure of LiRecDT7, the isoform LiRecDT1 was chosen as a template because the crystallized protein in Protein Data Bank (PDB) shows more identity with the novel isoform [de Giuseppe et al., 2011]. In addition, LiRecDT1 was used as a basis for comparison in all assays performed in the present work. As the images displayed in Figure 4 demonstrate, the overall structure of the novel isoform closely resembles the LiRecDT1 general outline, despite the differences observed in the primary structure. The presence and location of the disulfide bridges (Cys51–Cys57 and Cys53–Cys201), the Mg^{2+} binding site and the two catalytic histidine residues (His12 and His47) are conserved in LiRecDT7 model, which are components of the active-site pocket of spider venom PLDs [de Giuseppe et al., 2011].

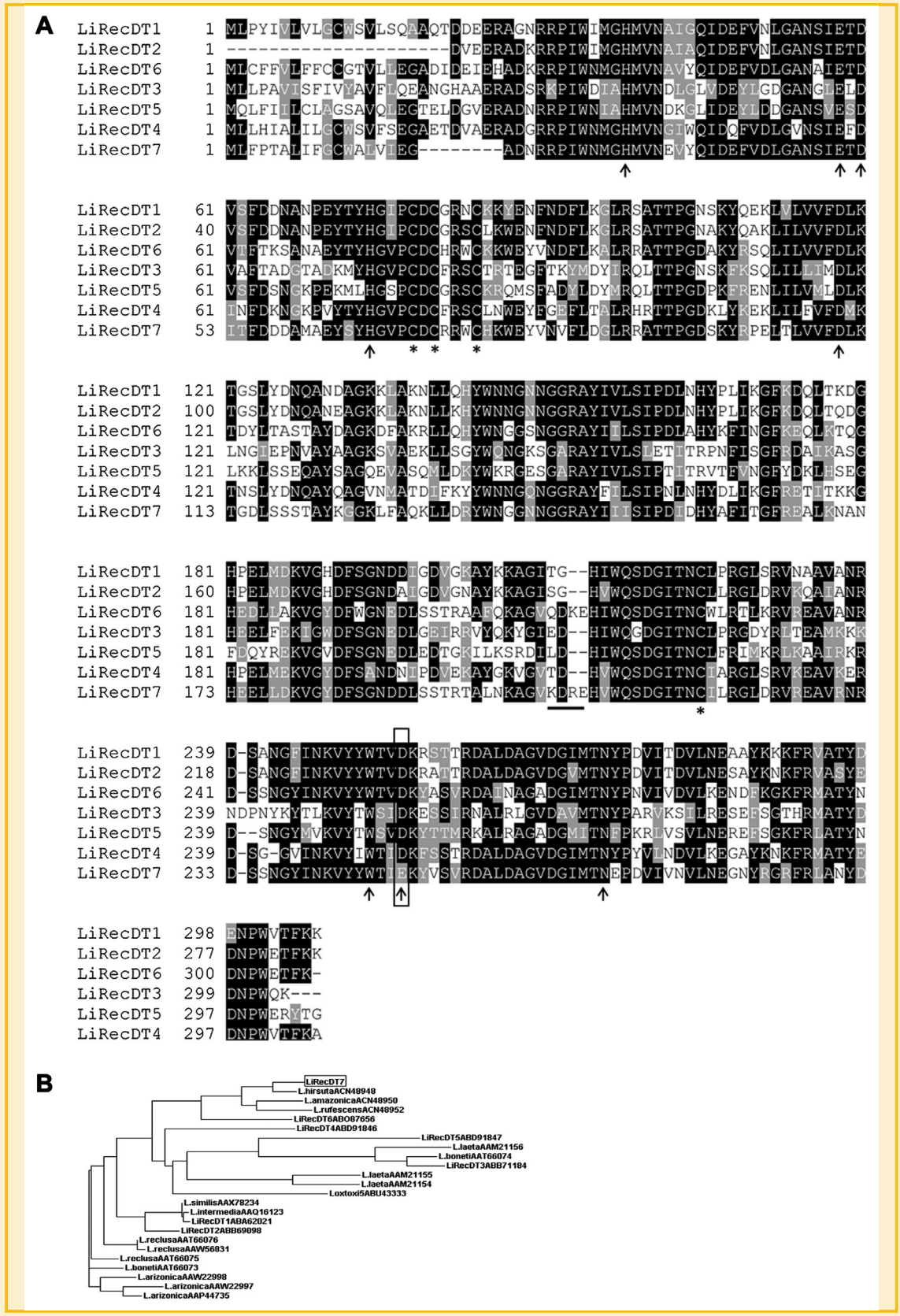

Fig. 2. Multiple alignment analysis of the cDNA-deduced amino acid sequence and the similarity relationship of described phospholipase-D with other phospholipase-D toxin family members from brown spider venoms. The sequences were aligned using the CLUSTAL W program (www.ebi.ac.uk/CLUSTAL). A: The black shaded regions show amino acid identity, the gray shaded regions show conservative substitutions, and the arrows point to amino acid residues of catalytic site of sphingomyelinases-D. The asterisks show cysteine residues. The conservative D223E substitution of LiRecDT7 is featured in the box. The line indicates the amino acids residues of the prominent loop. B: Similarity cladogram of the cloned phospholipase-D toxin members based on sequence alignment from GenBank data. The tree was constructed using the CLUSTAL program as described above. LiRecDT7 is highlighted in the box.

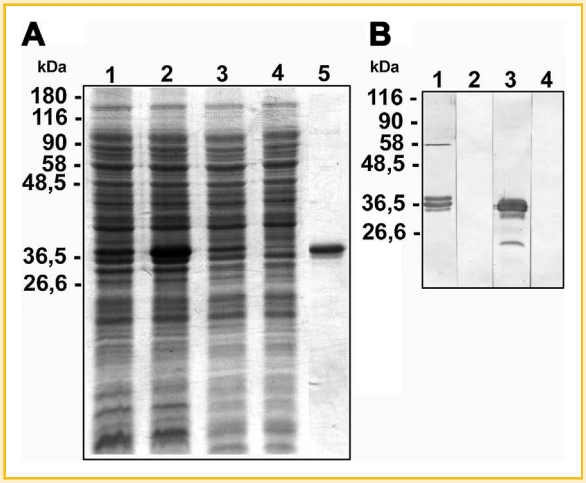

Fig. 3. Expression, purification, and immunological relationship of recombinant dermonecrotic toxin LiRecDT7 with other brown spider phospholipase-D members. A: The expression and purification of recombinant toxin was analyzed using 12.5% SDS–PAGE under reducing conditions and Coomassie blue dye staining. Lane 1 depicts *E. coli* SHuffle T7 Express *lysY* cells before 4 h of induction with 0.05 mM IPTG. Lane 2 shows the proteins of cells after induction with 0.05 mM IPTG. Lanes 3 and 4 depict the supernatant obtained through freeze, thawing and mechanical lysis in extraction buffer before and after affinity chromatography using a Ni^{2+}-NTA column, respectively. Lane 5 shows purified recombinant protein LiRecDT7. The molecular protein mass standards are shown on the left. B: Crude venom (lanes 1 and 2) and purified recombinant toxin LiRecDT7 (lanes 3 and 4; 2.5 μg) were separated using 12.5% SDS–PAGE under reducing conditions, transferred onto nitrocellulose membranes that were incubated with polyclonal antibodies against LiRecDT1 (lane 1) or polyclonal antibodies against whole venom toxins (lane 3). Lanes 2 and 4 indicate reactions in the presence of pre-immune serum (control for antibody specificity). The molecular mass markers are shown on the left.

However, it is possible to observe a significant variation in the contour of the loop located just behind the variable loop of both models. In the LiRecDT7 model, this loop is more prominent (directed toward the surface of the protein) compared with that in LiRecDT1, where this loop, although still prominent, is more positioned in the interior of the protein. As presented in Figure 4, this condition can be easily detected in LiRecDT7, as this loop follows the beginning of the near helix, while in LiRecDT1 this loop follows a different path, away from the near helix and toward the interior of the molecule.

In addition, in both models residue 233 is highlighted, illustrating the difference in the structures of aspartic acid (Asp) and glutamic acid (Glu); it is clear that Glu (LiRecDT7) has a bulkier side chain compared with to Asp (LiRecDT1), which protrudes towards the flexible loop.

SPHINGOMYELINASE AND HEMOLYTIC ACTIVITIES FOR LiRecDT7

To further assess the recombinant molecule functionality, LiRecDT7 was tested for its activity as a sphingomyelinase-D because the native brown spider dermonecrotic toxin and other LiRecDT's family members have sphingomyelinase-D activity. The sphingomyelinase activity was measured using an Amplex-R-Red Sphingomyelinase Assay Kit (as described in material and methods). LiRecDT1 (10 μg) was used as a positive control, and PBS was used as a negative control. Samples of 5, 10, and 20 μg of LiRecDT7 were tested in three subjects. As shown in Figure 5A, the recombinant LiRecDT7 toxin exhibited sphingomyelinase-D activity in a concentration-dependent manner. Nevertheless, as the graph depicts, LiRecDT7 exhibited lower sphingomyelinase-D activity than LiRecDT1.

In addition, we reported the hemolytic activity for *L. intermedia* recombinant phospholipases-D [Chaves-Moreira et al., 2009, 2011]. This hemolytic activity is attributed to the hydrolytic activity of toxins on erythrocyte membrane phospholipids, as toxins induce different degrees of hemolysis, which is proportional to sphingomyelinase-D activity. LiRecDT1H12A, a molecule without sphingomyelinase-D activity, also exhibits residual hemolytic activity [Chaves-Moreira et al., 2011]. Herein, we provide additional data suggesting the direct hemolysis activity induced though brown spider venom phospholipase-D. Figure 5B shows the hemolysis in human red blood cells incubated in serum-free medium containing different amounts of LiRecDT7 at 37°C. As observed, hemolysis occurred in a dose-dependent manner, indicating the direct hemolytic activity of LiRecDT7 in human erythrocytes.

SKIN LESIONS AND INFLAMMATORY RESPONSE EVOKED BY RECOMBINANT LiRecDT7

Because brown spider venom phospholipases-D possess dermonecrotic and inflammatory activities [da Silva et al., 2004; Appel et al., 2005; Chaim et al., 2011b], we evaluated the functionality of recombinant LiRecDT7. For this purpose, LiRecDT7 (10 μg) was injected intradermally into shaved rabbit skin. As a negative control, we used recombinant GFP (devoid of dermonecrotic activity), which was expressed and purified under the same conditions as those used for LiRecDT7. The macroscopic lesions were assessed 3, 6, and 24 h after injection, and the tissue samples were collected and histologically analyzed using a light microscope at 24 h after toxin exposure (Fig. 6). The animals that received recombinant toxin LiRecDT7 showed lesions with a deep erythema, a diffuse edema surrounding the lesion and a remarkable hemorrhage after 6 h. After 24 h, these signs were exacerbated, and an area of necrosis was observed surrounding the injection site. Gravitational spreading was not observed after the inoculation of LiRecDT7 compared with the clear spreading triggered by whole venom and LiRecDT1. The histopathological findings of skin biopsies after recombinant toxin exposure (LiRecDT7) showed a massive inflammatory response (Fig. 7C), with inflammatory cells (neutrophil leukocytes; Fig. 7D) diffusely spread within the dermis. Figure 7A,B shows panoramic views of sections of negative control and LiRecDT7, respectively, and clearly illustrate the edema triggered using the recombinant toxin, as evidenced by the length of the skin structures. As observed, the panoramic view of the tissue section after LiRecDT7 exposure is wider compared with that of the control, indicating an event induced through tissue edema. In addition, in Figure 7E it is possible to observe the disorganization of collagen fibers in the dense connective tissue inside the dermis, which also indicates edema. The images also depict areas of necrosis, including the degeneration of blood vessel walls (Fig. 7F,G). We also detected thrombus formation into dermal blood vessels after LiRecDT7 inoculation (Fig. 7H). Thus, the recombinant toxin LiRecDT7 is functional and a member of the family of dermonecrotic toxins.

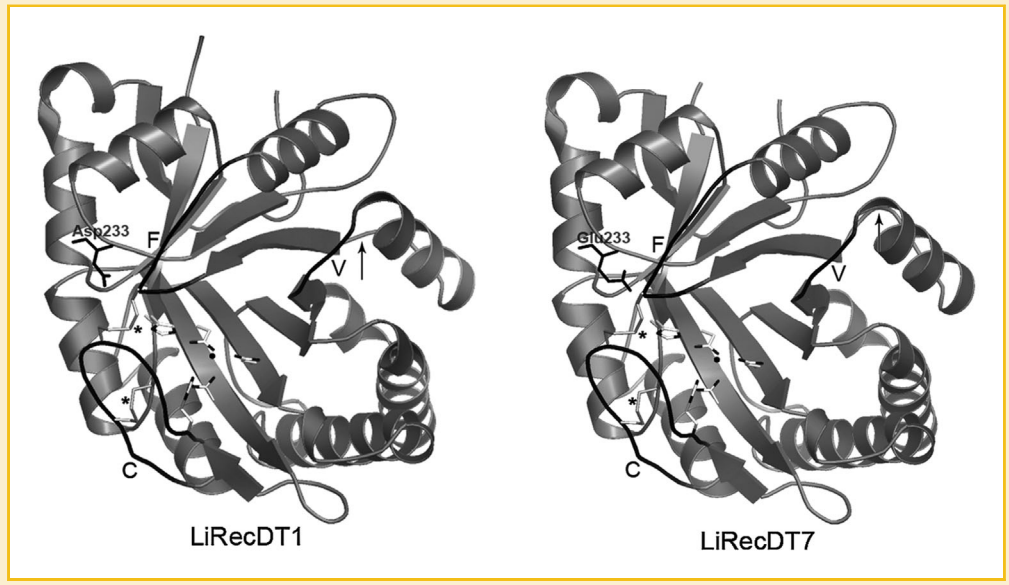

Fig. 4. Molecular modeling of LiRecDT1 and LiRecDT7. We used beta SWISS-MODEL to build a prediction of the three-dimensional structure of LiRecDT1 and LiRecDT7. Asp 233 in LiRecDT1 and Glu233 in LiRecDT7 are indicated. The black arrow indicates the significant variation of the loops in LiRecDT7 and LiRecDT1. The catalytic (C), variable (V), and flexible (F) loops are indicated. The asterisks indicate the disulfide bridges. The main amino acid residues involved in the coordination of the Mg^{2+} (represented by a sphere) in the catalytic site are highlighted (His12, Asp 32, Glu34, and His47).

RECOMBINANT TOXIN LiRecDT7 INCREASED VASCULAR PERMEABILITY

The data obtained from previous literature reported that brown spider phospholipase-D toxins increase vessel permeability [da Silveira et al., 2006, 2007c]. To examine whether LiRecDT7 could change vessel integrity and permeability in vivo, purified recombinant LiRecDT7, along with the appropriate negative control (PBS) and a positive control (LiRecDT1), was injected into the skin of mice that had been previously blue dye-perfused (Miles assay). The LiRecDT7 injection induced increased Evans blue extravasation compared with the negative control. Nevertheless, LiRecDT7 showed a lower activity compared with that of LiRecDT1 (Fig. 8).

DISCUSSION

Based on its ability to hydrolyze the phospholipid sphingomyelin into choline and C1P, previous studies have characterized brown spider venom phospholipase, also called "dermonecrotic toxin," as a sphingomyelinase-D molecule [da Silva et al., 2004]. However, the recent results obtained from lipid biochemical research have suggested that this toxin is a phospholipase-D enzyme because it degrades not only sphingophospholipids but also glycerophospholipids, generating C1P or lysophosphatidic acid (LPA) [Lee and Lynch, 2005; Chaim et al., 2011b; Chaves-Moreira et al., 2011]. It has been postulated that by degrading phospholipids and generating important lipid mediators, such as C1P or LPA, brown spider venom phospholipase-D toxin activates signaling pathways in different cells causing pathophysiological changes, such as inflammatory response, platelet aggregation, increased blood vessel permeability, hemolysis, and nephrotoxicity [Chaim et al., 2011b; Chaves-Moreira et al., 2011; Wille et al., 2013].

Currently, the idea of a family of similar molecules of phospholipase-D toxins in brown spider venom species is evident. This hypothesis was first suggested based on a biochemical characterization of *L. reclusa* venom, which contains four phospholipase-D isoforms [Futrell, 1992]. Additional studies revealed antigenic cross-reactivity for phospholipases-D from different brown spider venoms, including *L. gaucho*, *L. laeta*, and *L. intermedia* [Barbaro et al., 1996]. The biochemical and immunological analyses of *L. deserta* and *L. reclusa* venoms showed antigenic cross-reactivity and biochemical homologies (amino acid composition) for phospholipase-D toxins [Gomes et al., 2011]. Two phospholipase-D-like toxins were described in *L. gaucho* [Cunha et al., 2003] and four toxins were described in *L. boneti* venom [Ramos-Cerrillo et al., 2004]. Through proteomic analysis, several phospholipase-D isoforms were identified in *L. gaucho* venom [Machado et al., 2005], thereby confirming the idea that these molecules belong to a family of related toxins. Through molecular biology studies, the concept of an intra- and inter-species family of brown spider venom phospholipase-D was further confirmed by the cloning and expression of phospholipase-D toxins from a variety of *Loxosceles* spiders. Binford et al. [2005] reported three cDNA sequences for phospholipase-D toxins identified in *L. arizonica*. Chaim et al. [2006], da Silveira et al. [2006, 2007c], and Appel et al. [2008] cloned, expressed, and identified differential functionality for six related phospholipase-D molecules using a cDNA library obtained from the venom gland of *L. intermedia*. The transcriptional profile of the *L. intermedia* venom gland obtained through the construction of a wide cDNA library showed that members of phospholipase-D family represent 20.2% of the total

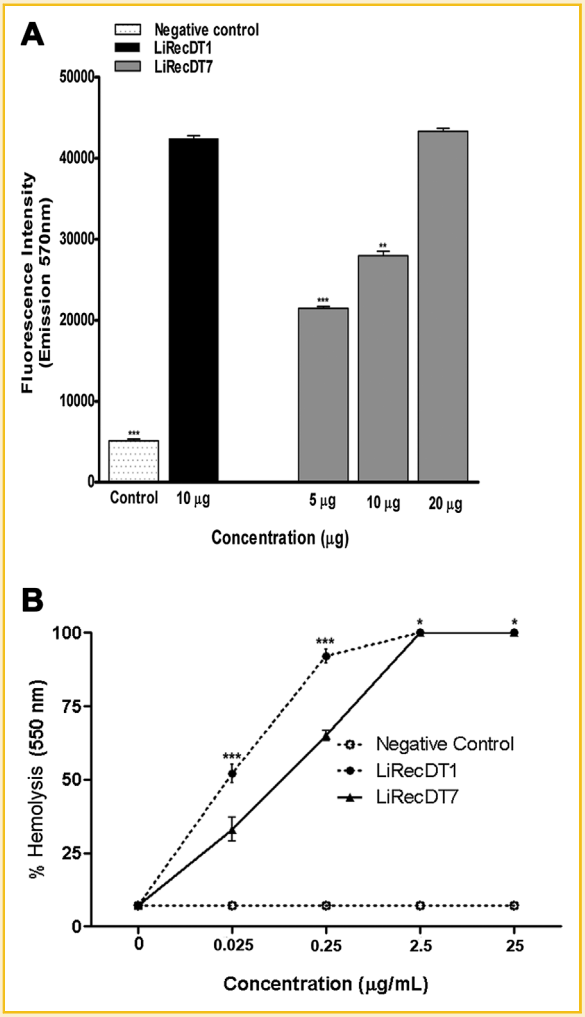

Fig. 5. Sphingomyelinase-D and hemolytic activities of LiRecDT7. A: Sphingomyelinase-D activities of recombinant dermonecrotic toxins LiRecDT1 and LiRecDT7 were evaluated using an Amplex Red Assay Kit at 37°C for 1 h, and the product of the reaction was determined at 550 nm. PBS was used as a negative control. Reactions used 5, 10, and 20 μg of LiRecDT7 and 10 μg of LiRecDT1 ($n = 5$). The means ± standard errors are shown, with significance levels $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ comparing activities of LiRecDT1 and LiRecDT7. B: Human erythrocytes suspended in TBS were incubated with different concentrations of LiRecDT7, or in the absence of toxin (negative control), for 24 h at 37°C. The absorbance values of the supernatants were measured at 550 nm, and the percentage of hemolysis was determined using the absorbance values induced with 0.1% (v/v) Triton X-100 as 100% hemolysis (positive control). The results represent the means of five experiments ± SEM. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

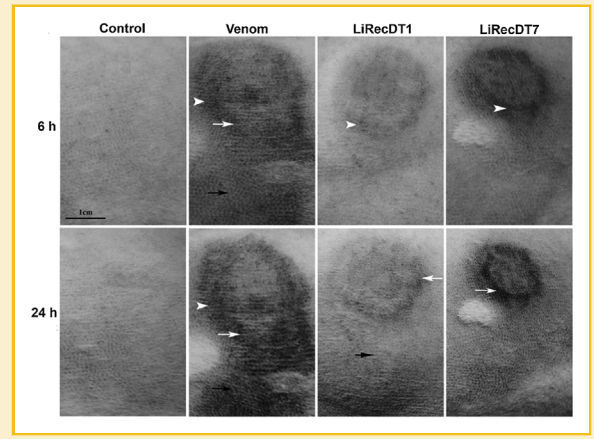

Fig. 6. Macroscopic and histological changes in rabbit skin exposed to whole venom and the recombinant toxins LiRecDT1, LiRecDT7 and negative control recombinant—Green Fluorescent Protein (GFP). Macroscopic visualization of dermonecrosis in rabbits intradermally injected with 10 μg of whole venom and the purified toxins LiRecDT1 and LiRecDT7. Lesions were observed at 6 and 24 h after the injections. The arrowhead indicates a hemorrhagic area surrounding the lesion. The white arrow indicates a necrotic area. The black arrows indicate gravitational spreading of lesions.

toxin-encoding transcripts [Gremski et al., 2010]. Brown spider venom toxins that have phospholipase-D activity are currently grouped into a family. It was postulated that the noxious effects induced by *Loxosceles* species crude venom might reflect the synergism among these related phospholipase-D toxins [Kalapothakis et al., 2007] and other components of venom, as the venom is a complex mixture containing additional constituents, such as insecticidal peptides, astacin-like metalloproteases, neurotoxins, serine proteases, venom allergen, translationally controlled tumor protein, hyaluronidases, serine protease inhibitors, and other components [Gremski et al., 2010].

Herein, we described the cloning, heterologous expression, affinity purification, and the functionality of a novel phospholipase-D "dermonecrotic toxin" family member from brown spider venom, strengthening the proteomic, immunological, and molecular biology data previously reported. The recombinant toxin identified was designated LiRecDT7 (GenBank Accession no. KC237286). The name is a reference to other previously identified brown spider (*L. intermedia*) venom phospholipase-D isoforms (LiRecDT1, LiRecDT2, LiRecDT3, LiRecDT4, LiRecDT5, and LiRecDT6) [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Appel et al., 2008].

Initially, our experimental strategy for cloning this novel member of brown spider venom phospholipase-D toxin cDNA was based on the random sequence analysis obtained from a cDNA library of *L. intermedia* venom gland [Gremski et al., 2010] using a BLAST search for similarities with previous cloned phospholipase-D toxins and 5′ RACE amplification to obtain the complete cDNA sequence.

The complete sequence of this novel brown spider phospholipase-D includes a signal peptide and a mature protein with high similarity to other previously reported brown spider venom phospholipase-D molecules [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Kalapothakis et al., 2007; Appel et al., 2008]. The deduced LiRecDT7 protein displayed an amino acid sequence identity of 63% compared with LiRecDT1. The similarities between LiRecDT7 and the other isoforms of *L. intermedia* varied showing the highest similarity with LiRecDT6 (71%) and the lowest similarity with LiRecDT3 (45%). Compared with phospholipase-D toxins from other *Loxosceles* species, the highest sequence similarities to LiRecDT7 were observed for sphingomyelinase-D alphaIV2 of *L. hirsuta* (ACN48948—91% amino acid identity). Binford et al. [2009] showed that recent gene duplications are apparent in groups of closely related species, which is

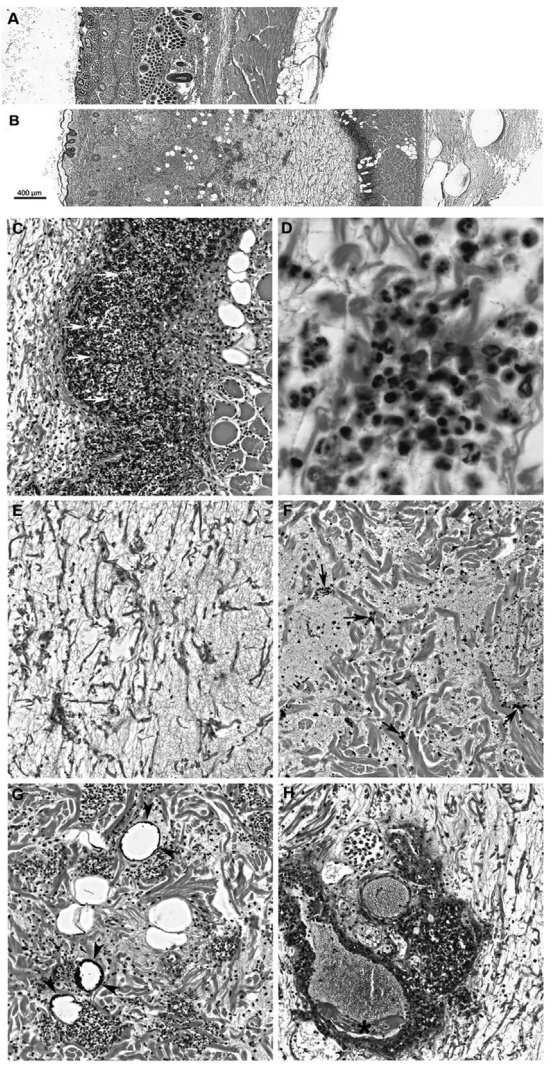

Fig. 7. Histopathology features of rabbits' skin 24 h following LiRecDT7 exposure. Light microscopy analysis of sections of dermonecrotic lesions stained with H&E. A,B: Panoramic views of sections of negative control recombinant—green fluorescent protein (GFP)—and LiRecDT7, respectively, clearly demonstrating the edema triggered by the recombinant toxin. At this stage, there was a massive inflammatory response (white arrows) (C), with the presence of neutrophils in the dermis (D), the disorganization of collagen fibers (E), areas of necrosis (black arrows; F), including the degeneration of blood vessel walls (black arrowheads; G), and thrombus formation into dermal blood vessels (asterisk; H). Magnification of panoramic views: 15×; (C,E–H) 100×; (D) 630×.

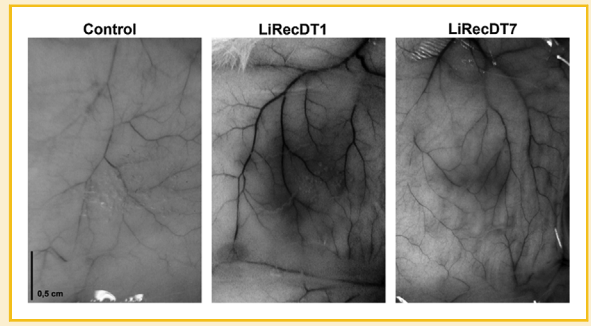

Fig. 8. Effect of LiRecDT7 on vascular permeability of skin vessels. The mice were administered with intradermal injections (10 μg) of LiRecDT7, LiRecDT1, and PBS (control for baseline permeability level; n = 5 per treatment). The photographs show increased dye leakage after recombinant toxin LiRecDT1 and LiRecDT7 exposure compared with minimal vessel permeability due to the negative control PBS.

the case for *L. intermedia* and *L. hirsuta*, members of the *spadicea* group. Thus, the high similarity observed between LiRecDT7 and sphingomyelinase-D alphaVI2 certainly reflects the closeness among the species. Some authors argue that to mantain effectiveness against preys and predators, the genes encoding venom peptides and proteins have undergone multiple duplication events. The duplicated genes, in turn, acquire related or even novel functions through adaptive evolution [Ma et al., 2012].

Moreover, LiRecDT7 contains the conserved amino acid residues involved in catalysis or metal ion coordination, which are important for phospholipase-D activity [Murakami et al., 2006], except for a punctual and conservative substitution at position 233 in the catalytic site. While all *L. intermedia* phospholipase-D isoforms contain a conserved an Asp residue in this position, LiRecDT7 has a Glu (D233E).

Structural analyses of LiRecDT1 show that the catalytic site of this enzyme is formed by two histidines at positions 12 and 47 and an Mg^{2+} ion. This catalytic site is hexacoordinated by Glu32, Asp34, and Asp91, a water molecule and two PEG4 oxygens [de Giuseppe et al., 2011]. This ion coordination is stabilized through hydrogen bonds formed by Asp34, Asp52, Trp230, Asp233, Asn252, and Gly480 [Murakami et al., 2005, 2006; de Andrade et al., 2006; de Giuseppe et al., 2011].

Asp is frequently involved in the formation of hydrogen bonds that assist in stabilizing protein structures. This residue might also be involved in active or binding sites for interactions with other proteins. Glu is also a charged amino acid. However, Asp has a less bulky side chain and is less flexible when located in the interior of a protein. These properties confer a slight preference for the inclusion of Asp in protein active sites, as is observed with the Asp233 residue in *Loxosceles* phospholipases-D, which is highly conserved. This phenomenon has also been observed in the classic example of the active site of serine proteases, whose mechanism is based in the amino acid triad Asp-His-Ser [Betts and Russell, 2003]. In this context, the substitution of Asp for Glu (Asp → Glu) is quite rare, although it is possible that Glu in the position 233 might play a similar role stabilizing the ion coordination structure of LiRecDT7, consistent with the observed biochemical and biological properties of this toxin.

Several other structural and biochemical characteristics provide confirmation of LiRecDT7 as a member of the brown spider phospholipase-D venom toxin family. First, the molecular mass of LiRecDT7 (36 kDa) and the calculated isoelectric point from the deduced amino acid sequence of the mature protein (pI 5.94) are similar to those described for other phospholipase-D members (ranging from 31 to 33 kDa and pI 5.3 to 8.7) [Kalapothakis et al., 2007]. Using immunological approaches, LiRecDT7 cross-reacted with antibodies against LiRecDT1, confirming antigenic homology

with LiRecDT1. Moreover, antibodies for crude venom toxins also cross-reacted with recombinant LiRecDT7, demonstrating that the structures of crude venom proteins are similar to LiRecDT7 and that LiRecDT7 shows sequence/epitope similarities with native venom toxins. Taken together, these results demonstrate that the phospholipase-D epitopes are strong antigenic determinants.

Full length LiRecDT7 was heterogeneously expressed in *E. coli* SHuffle T7 Express *lysY* cells and purified using single step affinity chromatography. LiRecDT7 was eluted in a pure form as visualized through SDS–PAGE. Using this approach, it was possible to obtain purified material for biochemical and functional analyses.

The native brown spider venom phospholipase-D toxins and previously described LiRecDT1 to 6 isoforms [Chaim et al., 2006; da Silveira et al., 2006, 2007c; Appel et al., 2008] exhibit sphingomyelinase-D activity. As expected, the amino acid alignment and similarity analysis demonstrated the homology of LiRecDT7 with other brown spider phospholipase-D isoforms, and LiRecDT7 demonstrated sphingomyelinase-D activity through the generation of choline in a concentration-dependent manner. The substitution of the Asp residue with Glu at position 233 in the catalytic site did not abolish the sphingomyelinase-D activity of isoform LiRecDT7, although a reduction in the enzymatic activity was observed compared with LiRecDT1. Nevertheless, these results confirm the biochemical nature of LiRecDT7 as a sphingomyelinase-D and strongly suggest functionality for this LiRecDT isoform. However, with our results, it is not possible to infer whether this novel isoform is able to hydrolyze other phospholipids as observed for other members of this family [Chaim et al., 2011b]. Further studies specifically focusing on these activities of LiRecDT7 are needed to identify such aspects. As previously reported, all pathophysiological events triggered by brown spider venom recombinant isoforms, such as nephrotoxicity, dermonecrosis, inflammatory response, mice lethality and hemolysis are dependent on phospholipase-D catalysis [Kusma et al., 2008; Paludo et al., 2009; Chaim et al., 2011a; Chaves-Moreira et al., 2011].

The data obtained from the literature have described brown spider venom phospholipases-D as remarkable inducers of necrotic skin lesions and inflammatory response. Due to these effects, brown spider venom phospholipase-D toxins are also referred to as "dermonecrotic toxins." Indeed, the hallmark of accidents following brown spider bites is dermonecrosis with gravitational spreading surrounding the bite site; thus, loxoscelism is also referred to as necrotic arachnidism or gangrenous arachnidism [da Silva et al., 2004; Senff-Ribeiro et al., 2008]. Skin lesions triggered by brown spider phospholipase-D toxins are the consequence of a massive inflammatory response observed in the epidermis and dermis. This event is histopathologically referred to as "aseptic coagulative necrosis" and is a consequence of dysregulated endothelial cell-dependent neutrophil activation [Ospedal et al., 2002]. As previously described for other LiRecDT isoforms, LiRecDT7 demonstrated necrotic and inflammatory activities, providing further evidence of the activity of this toxin. LiRecDT7 induced noxious responses upon injection in rabbit skin (a macroscopic lesion), with swelling, erythema, hemorrhage, ischemia, and necrosis. Histological studies of samples collected from macroscopic lesions at 24 h after toxin exposure provided additional evidence for LiRecDT7 as an inducer of the inflammatory response. The observed histopathological changes included diffuse dermal edema, proteinaceous exudation and a massive and diffuse aggregation of leukocytes into the dermis. In addition, LiRecDT7 induced tissue necrosis, including degeneration of blood vessel walls. The novel phospholipase-D isoform also triggered thrombus formation into dermal blood vessels. These events are consistent with the literature data and resemble the observations reported after crude venom exposure [Veiga et al., 2001; Ospedal et al., 2002; da Silva et al., 2004].

Furthermore, the functionality of LiRecDT7 was also supported through the observed increase in capillary permeability in mice after treatment with purified recombinant toxin. This event has been described in *L. intermedia* whole venom, native brown spider venom phospholipase-D toxins and other LiRecDT isoforms [da Silva et al., 2004; da Silveira et al., 2007c; Ribeiro et al., 2007; Appel et al., 2008]. Notably, the increase in blood vessel permeability triggered by LiRecDT1 was more prominent than that induced by LiRecDT7. As described for other LiRecDTs, this ability is associated with the level of enzymatic activity of the isoform, as observed for LiRecDT3 and LiRecDT5, which show lower sphingomyelinase-D activity than LiRecDT1 and also trigger a less prominent increase in blood vessel permeability [da Silveira et al., 2006, 2007c]. Apparently, through the induction of inflammatory response and leukocyte infiltration into the dermis of mice [Sunderkotter et al., 2001], phospholipase-D toxins increase vessel permeability. In addition, a direct phospholipase-D effect upon endothelia, inducing endothelial cell activation and cytotoxicity, as previously reported for brown spider crude venom [Veiga et al., 2001; Zanetti et al., 2002; Paludo et al., 2009] increases vessel permeability through a disruption of the blood vessel wall.

LiRecDT7 also induced the direct lysis of human erythrocytes. The hemolytic effect of brown spider venom has been demonstrated through the clinical and laboratory features observed in accident victims. These features include hematuria, hemoglobinuria, elevated creatine kinase levels, proteinuria and shock [Kusma et al., 2008]. We have previously described the direct hemolytic effect of LiRecDT1 upon erythrocytes of different animal sources. This activity is not dependent on the ABO or Rhesus systems, but rather is dependent on the animal species, as human, sheep, and rabbit erythrocytes were lysed, but erythrocytes from horses were less severely damaged after toxin treatment, and these results depend upon the membrane composition of the cells. This event is also dependent on the catalytic activity of the phospholipase-D, as a site-directed mutation in the catalytic site (H12A) completely abolished the hemolytic effect of the mutated isoform LiRecDT1H12A [Chaves-Moreira et al., 2009]. The results described herein using LiRecDT7, demonstrate the direct hemolytic effect of this recombinant phospholipase-D on human erythrocytes. Toxin-dependent hemolysis occurs in a concentration-dependent manner, supporting the specificity of this effect.

Based on the crystallography results obtained using recombinant phospholipase-D toxin isoforms from *L. laeta* and *L. intermedia* venom and the structural and sequence alignment comparison data [Murakami et al., 2006; de Giuseppe et al., 2011], a classification system was proposed, which considers the phospholipase-D activity of these toxins. According to the authors, brown spider venom dermonecrotic toxins can be divided into two categories: class I molecules, containing a single intrachain disulfide bond and one

extended hydrophobic loop (*L. laeta* isoform) and class II molecules, containing an additional disulfide bond linking the catalytic loop to a second flexible loop. The class II phospholipases can be subdivided into classes IIa and IIb according to their ability, or lack thereof, to hydrolyze sphingomyelin, respectively. Based on this classification, LiRecDT7 belongs to class IIa, as this isoform possesses two putative disulfide bonds (Cys51–Cys57 and Cys53–Cys201) and is able to hydrolyze sphingomyelin.

The molecular modeling data also support the classification of LiRecDT7 as a class II sphingomyelinase-D, as it revealed the presence of two disulfide bridges in the putative structure of this novel isoform, as observed in LiRecDT1 model. The 3D modeling of LiRecDT7 also demonstrated that the structural basis for Mg^{2+} ion coordination and the two catalytic histidine residues (His12 and His47), which play key roles in the active-site pocket of spider venom phospholipases-D, is maintained compared with LiRecDT1.

In LiRecDT1, these two catalytic histidine residues (His12 and His47), are supported through a network of hydrogen bonds between Asp34, Asp52, Trp230, Asp233, and Asn252. Because LiRecDT7 possesses a substitution of Asp233 to Glu233, this residue is highlighted in the 3D modeling of both isoforms (LiRecDT1 and LiRecDT7) to illustrate this natural mutation. It is clear that the side chain of Glu in LiRecDT7 is bulkier than the side chain of Asp in LiRecDT1. There is also an obvious variation in the positioning of the side chain of these residues, as Glu in LiRecDT7 protrudes toward the flexible loop. It is not possible to infer whether these differences affect the network of hydrogen bonds between this residue and the two catalytic histidine residues. Indeed, this natural mutation did not abolish the enzymatic and biological actions of this novel isoform. The molecular modeling of LiRecDT7 also revealed that one of the loops, located near the variable loop, is more prominent (directed toward the surface of the molecule) compared with the same loop in LiRecDT1. When comparing the primary structure of this specific region, we observed the presence of lysine (207), Asp (208), arginine (209) and Glu (210) residues in LiRecDT7. These amino acids generally prefer to reside on the surface of the protein. Lysine and arginine are also frequently involved in *salt-bridges* where they pair with a negatively charged Asp or Glu to stabilize hydrogen bonds that are important for protein stability [Betts and Russell, 2003]. These features might explain the prominent configuration of this loop in LiRecDT7. In addition, as shown in Figure 2, LiRecDT1 has only two amino acid residues in this region. Thus, the presence of two additional residues in this region of LiRecDT7 might also explain the presence of a longer loop in this isoform. Moreover, Figure 2 also shows that the only isoform containing the four conserved amino acid residues in this region is LiRecDT6, although it is not possible to infer a direct relation concerning their structures based on this observation.

The results presented herein, demonstrate that LiRecDT7 possesses an intermediate ability for sphingomyelin hydrolysis and biological activities compared with LiRecDT1. A putative explanation for the differences described could be inferred from a mutation in the catalytic site (D233E) that, despite involving amino acids from the same characteristics (negative charged residues), in some way could destabilize and disorganize the catalytic site, affecting enzyme/substrate interactions in the catalytic cleft as the Glu residue contains an additional CH_2 group. However, it is not possible to infer that D233E substitution alone is responsible for the observed differences, as some members of the phospholipase-D family in *L. intermedia* exhibit lower sphingomyelinase-D activity, even with all amino acids of the catalytic site conserved (e.g., LiRecDT3, LiRecDT4 and LiRecDT5) [da Silveira et al., 2006, 2007c]. As observed for these mentioned isoforms, LiRecDT7 also possesses several substitutions in the amino acid residues neighboring the catalytic site. As previously proposed [da Silveira et al., 2006] these residues might be involved in the stabilization and organization of the catalytic site or even in the synergistic domains of these toxins. Thus, in addition to the specific substitution present in LiRecDT7, these differences might also explain the variations in functionality between these isoforms.

Venom toxin molecules have recently been used to investigate molecular and cellular mechanisms, as models for the design of novel drugs or even for diagnostic or therapeutic uses [Senff-Ribeiro et al., 2008; Chaim et al., 2011a; Horta et al., 2013]. The development of a novel recombinant *Loxosceles* phospholipase-D toxin can provide an agonist molecule as an additional tool to study the inflammatory response or to design and identify antagonist molecules using co-crystallization techniques and X-ray diffraction procedures. Additionally, this novel phospholipase-D can be used as a tool in biochemical lipid research protocols or as a recombinant antigen for serum therapy applications.

In summary, we have identified a novel brown spider venom phospholipase-D "dermonecrotic toxin" family member. This molecule, referred to as LiRecDT7, was cloned, heterogeneously expressed and purified. LiRecDT7 degraded sphingomyelin to generate choline in a concentration-dependent manner, induced dermonecrosis in rabbit skin and increased inflammation in the dermis of these animals. LiRecDT7 also increased vascular permeability in mice and induced direct hemolysis in human erythrocytes. Together, these results provide new insights into loxoscelism, contribute to the understanding of venom phospholipase-D and present the possibility of applying venom toxins as biotechnological tools for lipid research.

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