

Functional analysis of DM64, an antimyotoxic protein with immunoglobulin-like structure from *Didelphis marsupialis* serum

Surza L. G. Rocha¹, Bruno Lomonte³, Ana G. C. Neves-Ferreira¹, Monique R. O. Trugilho¹, Inácio de L. M. Junqueira-de-Azevedo^{4,5}, Paulo L. Ho^{4,5}, Gilberto B. Domont², José M. Gutiérrez³ and Jonas Perales¹

¹Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil; ²Departamento de Bioquímica, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; ³Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; ⁴Centro de Biotecnología, Instituto Butantan, and ⁵Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

Bothrops snake venoms are known to induce local tissue damage such as hemorrhage and myonecrosis. The opossum *Didelphis marsupialis* is resistant to these snake venoms and has natural venom inhibitors in its plasma. The aim of this work was to clone and study the chemical, physicochemical and biological properties of DM64, an antimyotoxic protein from opossum serum. DM64 is an acidic protein showing 15% glycosylation and with a molecular mass of 63 659 Da when analysed by MALDI-TOF MS. It was cloned and the amino acid sequence was found to be homologous to DM43, a metalloproteinase inhibitor from *D. marsupialis* serum, and to human α_1 B-glycoprotein, indicating the presence of five immunoglobulin-like domains. DM64 neutralized both the *in vivo* myotoxicity and the *in vitro* cytotoxicity of myotoxins I (mt-I/Asp49) and II (mt-II/Lys49) from *Bothrops asper* venom. The inhibitor formed noncovalent

complexes with both toxins, but did not inhibit the PLA₂ activity of mt-I. Accordingly, DM64 did not neutralize the anticoagulant effect of mt-I nor its intracerebroventricular lethality, effects that depend on its enzymatic activity, and which demonstrate the dissociation between the catalytic and toxic activities of this Asp49 myotoxic PLA₂. Furthermore, despite its similarity with metalloproteinase inhibitors, DM64 presented no antihemorrhagic activity against *Bothrops jararaca* or *Bothrops asper* crude venoms, and did not inhibit the fibrinogenolytic activity of jararhagin or bothrolysin. This is the first report of a myotoxin inhibitor with an immunoglobulin-like structure isolated and characterized from animal blood.

Keywords: *Didelphis marsupialis*; inhibitor; myotoxin; phospholipase; snake venom.

Envenomation by snakes of the Viperidae family usually causes local tissue damage such as edema, hemorrhage and myonecrosis [1,2], which are poorly neutralized by conventional antivenom serotherapy. In severe cases, these local effects may lead to permanent tissue loss, disability or amputation [3,4]. Myonecrosis causes irreversible cell damage to skeletal muscle fibers due to the action of venom components that directly affect the integrity of their plasma

membrane [5]. In addition, myonecrosis in Viperidae envenomation can be secondary to the ischemia that results from the action of venom hemorrhagic metalloproteinases [6].

At least three groups of snake venom components have been found to produce direct myotoxic effects: (a) highly basic single-chain polypeptides of 42–45 amino acid residues cross-linked by three disulfide bridges, such as myotoxin a and crotamine, which are not enzymatically active and are typically found in *Crotalus* [5] and *Sistrurus* [7] venoms, (b) 12–16 kDa phospholipase A₂ (PLA₂) myotoxins classified as either class I (elapid and hydrophid snake venoms) or class II (viperid/crotalid venoms). Some class II PLA₂ myotoxin variants present a drastically reduced or lack of catalytic activity due to substitutions of critical residues in the calcium-binding loop, particularly at position 49, where an aspartic acid is replaced by lysine (PLA₂-Lys49). In few cases, the aspartic acid is replaced by serine (PLA₂-Ser49), which does not necessarily impair enzymatic activity. These PLA₂ proteins have been detected in venom as monomeric, dimeric or multimeric forms. (c) Cardiotoxins are basic polypeptides present in some elapid venoms, which affect the integrity of the sarcolemma by a nonenzymatic mechanism [7,8].

In most cases, the resistance of animals to snake venoms, mainly exhibited by snakes and certain mammals (hedgehog, opossum, mongoose), can be explained by the presence of neutralizing protein factors in their blood which inhibit

Correspondence to J. Perales, Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, Fiocruz, 21045-900 Rio de Janeiro, Brazil. Tel.: + 55 21 2562 0755; Fax: + 55 21 2590 9490; E-mail: jperales@ioc.fiocruz.br

Abbreviations: BaMIP, *Bothrops asper* myotoxin inhibitory protein; Bav, *Bothrops asper* venom; Bju, *Bothrops jararaca* venom; CgMIP, *Cerrophidion godmani* myotoxin inhibitory protein; CK, creatine kinase; CNBr, cyanogen bromide; LDH, lactate dehydrogenase; mt, myotoxin; PLA₂, phospholipase A₂; PLI, PLA₂ inhibitor; SVMF, snake venom metalloproteinase; TFMS, trifluoromethanesulfonic acid.

Enzymes: bothrolysin (EC 3.4.24.50); creatine kinase (EC 2.7.3.2); jararhagin (EC 3.4.24.73); lactate dehydrogenase (EC 1.1.1.27); myotoxin I (EC 3.1.1.4).

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important toxic components [9,10]. These factors are either metalloproteinase inhibitors (antihemorrhagic factors) or phospholipase A₂ inhibitors (PLIs) (antineurotoxic and/or antimyotoxic factors) [11,12]. The PLIs isolated from snake plasma have been classified into three groups based on their structural characteristics: PLI α contains carbohydrate-recognition-like domains also found in C-type lectins and mammalian M-type PLA₂ receptors; the only PLI β isolated so far has 33% identity to human leucine-rich α_2 -glycoprotein, a serum protein of unknown function; the PLI γ group is characterized by the presence of two tandem patterns of cysteine residues constituting two internal three-finger shaped motifs typical of urokinase-type plasminogen activator receptor (u-PAR) and cell surface antigens of the Ly-6 superfamily [13,14].

The first well characterized PLI with antimyotoxic activity was isolated from the blood of *Bothrops asper* [15]. BaMIP is an acidic oligomeric glycoprotein of 120 kDa composed of five 23–25 kDa subunits. Its N-terminal sequence is similar to several PLI α , therefore suggesting the presence of a carbohydrate-recognition-like domain in the inhibitor structure. In addition to its PLA₂ inhibitory activity against the basic myotoxins I and III from *B. asper* venom, BaMIP also inhibited the myotoxic, edematogenic and cytolytic activities of all four *B. asper* myotoxins isoforms (I–IV), irrespective of their PLA₂ activity.

Two serum myotoxin inhibitors, named CgMIP-I (γ -type) and CgMIP-II (α -type), were isolated, characterized and cloned from another viperid snake (*Cerrophidion godmani*) [16]. These inhibitors are acidic glycoproteins of 110 kDa (CgMIP-I) and 180 kDa (CgMIP-II) composed of 20–25 kDa subunits. CgMIP-I specifically neutralized the PLA₂ and the myotoxic, edema-forming and cytolytic activities of the enzymatically active myotoxin I from *C. godmani*, whereas CgMIP-II selectively inhibited the toxic properties of the enzymatically inactive myotoxin II. No PLI or antimyotoxic protein from mammals has been isolated so far.

Previous results have shown that the crude serum, as well as partially purified serum fractions from South American Didelphidae, inhibit the release of sarcoplasmic enzymes from skeletal muscle induced by *Bothrops jararacussu* venom [17]. Muscular and skin necroses induced by several *Bothrops* venoms were also inhibited [18,19]. However, because most of these studies were done with crude venoms, it is difficult to differentiate between direct myotoxic effect and muscle damage secondary to hemorrhage. At least two antitoxic proteins, named DM40 and DM43, have already been isolated from *Didelphis marsupialis* serum and characterized as inhibitors of hemorrhagic snake venom metalloproteinases [20]. The aim of this work was the chemical, physicochemical and functional characterization as well as the molecular cloning and sequencing of the antimyotoxic protein present in *D. marsupialis* serum.

EXPERIMENTAL PROCEDURES

Materials

DEAE-Sephacel, Hitrap® NHS-activated affinity column, Superdex 200 and HiPrep® Sephacryl S-200 columns, calibration standards for SDS/PAGE, gel filtration and isoelectric focusing, as well as oligo(dT)-cellulose columns

and *EcoRI* adapters were from Amersham Pharmacia Biotech, Sweden. Ampholytes (Bio-Lyte 3/10) were from Bio-Rad Laboratories, USA. Cyanogen bromide (CNBr) was from K & K Laboratories, USA. Sequencing grade endoproteinase Lys-C was from Boehringer Mannheim, Germany. Trizol reagent, the Superscript plasmid system and plasmid specific primers (M13F-cccagtcacgacgtttaaaccg- and M13R-agcggataacaatttcacacagg) were from Life Technologies, Inc. All other chemicals were of analytical grade or higher quality.

Animals, venoms, and toxins

D. marsupialis specimens were caught in the outskirts of Rio de Janeiro City, Brazil, under a license of the Brazilian Environmental Institute (IBAMA). Wistar rats and Swiss-Webster mice were from the Oswaldo Cruz Foundation Animal Breeding Unit. All experiments with animals were performed in accordance with the ethical standards of the International Society on Toxinology [21]. Lyophilized *B. jararaca* venom (Bjv) was from the Army Biology Institute, RJ, Brazil and lyophilized *B. asper* venom (Bav) was from Clodomiro Picado Institute, University of Costa Rica, San José, Costa Rica. Myotoxins I and II were isolated from *B. asper* venom as described previously [22,23], while jararhagin and bothrolysin were purified from *B. jararaca* venom according to Neves-Ferreira *et al.* [24].

Purification of DM64

Opossum serum was obtained from blood collected by cardiac puncture as described previously [25]. Serum was dialyzed for 24 h at 4 °C against the column equilibration buffer. After centrifugation, the supernatant was fractionated on a DEAE-Sephacel column (2.6 × 17 cm) equilibrated with 0.01 M sodium acetate buffer, pH 3.7. Elution was carried out isocratically with the equilibration buffer, followed by a linear NaCl gradient from 0.15–0.5 M in this same buffer at a flow rate of 0.5 mL·min⁻¹. The heterogeneous DM64 fraction was pooled, precipitated with ammonium sulfate at 80% saturation, dissolved in 0.02 M sodium phosphate, pH 7.0, and dialyzed against the same buffer. After centrifugation, the supernatant was isocratically fractionated, using this last buffer, on a Hitrap® NHS-activated affinity column (1 mL) containing myotoxin I from *B. asper* immobilized according to the manufacturer's instructions. The bound fraction was eluted with 0.1 M glycine/HCl, pH 2.7, and collected over 1 M Tris to neutralize the pH, at a flow rate of 1 mL·min⁻¹. Homogeneous DM64 was pooled, dialyzed against 0.01 M ammonium carbonate, lyophilized and stored at -20 °C. Protein contents were determined by the Lowry method [26] using BSA as a standard. Routinely, in all inhibition assays, the toxins and the inhibitor were mixed and incubated for 30 min at 37 °C.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in 12% separating and 4% stacking gels [27], using the Mini-Protean II system (Bio-Rad Laboratories, USA). Protein bands were stained with Coomassie Blue R-250. Molecular mass standards were phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin

(43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Molecular mass

DM64 molecular mass was determined by MALDI-TOF MS on a Voyager DE-PRO instrument (Perseptive Biosystems). The matrix used was 3,5-dimethoxy-4-hydroxy cinnamic acid. To determine the quaternary structure of DM64, molecular masses were also estimated by SDS/PAGE [27] following the method of Weber and Osborn [28] and by gel filtration on a Sephacryl S-200 column (1.6×60 cm) eluted at $0.5 \text{ mL} \cdot \text{min}^{-1}$ with 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0, and also on a Superdex 200 column (1.0×30 cm) eluted at $0.5 \text{ mL} \cdot \text{min}^{-1}$ with 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0, either in the presence or absence of 6 M guanidine-HCl. Molecular mass standards were BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa). Di-BSA (134 kDa) present in the BSA standard was also used as marker.

Chemical deglycosylation

DM64 was chemically deglycosylated with anhydrous trifluoromethanesulfonic acid (TFMS) using the GlycoFree kit k-500 from Oxford GlycoSystems, USA and submitted to SDS/PAGE. Human α -1 acid glycoprotein was used as deglycosylation control. Glycoproteins were visualized using periodic acid-Schiff stain.

Isoelectric focusing

DM64 was electrofocused using a Mini IEF system (Bio-Rad Laboratories, USA) and thin-layer polyacrylamide gels prepared according to the manufacturer's instructions, using wide range ampholytes (pH 3–10). pI calibration standards were amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β -lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (acidic, 6.85; basic, 7.35), lentil lectin (acidic, 8.15; middle, 8.45; basic, 8.65) and trypsinogen (9.30).

Amino acid sequence

DM64 was reduced, S-pyridylethylated and either directly N-terminal sequenced or cleaved with CNBr [29]. The CNBr peptides were isolated by Tricine-SDS/PAGE [30], transferred to a poly(vinylidene difluoride) (PVDF) membrane and submitted to Edman degradation on a Shimadzu PSQ-23A protein sequencer. A sample of DM64 was also reduced, alkylated with N-isopropyliodoacetamide [31] and digested with endoproteinase Lys-C. N-terminal sequence of the Lys-C digestion peptides purified by RP-HPLC [24] was performed on an Applied Biosystems 494 Procise instrument. DM64 partial sequence was used to scan the GenBank, SwissProt and PIR databases for similar sequences with the BLAST program [32].

Isolation of mRNA from liver

One specimen of *D. marsupialis* was sacrificed and its liver was immediately removed and kept in liquid nitrogen. For

total RNA extraction, the Trizol reagent was employed according to the manufacturer's protocol. A column of oligo(dT)-cellulose was used for mRNA purification.

cDNA library construction

The cDNAs were synthesized from $5 \mu\text{g}$ of mRNA using the Superscript plasmid system for cDNA synthesis and cloning linked to *EcoRI* adapters, selected by size (greater than 1000 bp) in agarose gel electrophoresis and directionally cloned in pGEM11Zf+ plasmid (Promega) at *EcoRI/NotI* sites [33]. *Escherichia coli* DH5 α cells were transformed with the cDNA library plasmids and then plated on a 2YT agarose plate containing $100 \mu\text{g} \cdot \text{mL}^{-1}$ ampicillin [34].

DNA sequencing

DNA sequencing was performed on a Perkin-Elmer 377, ABI Prism DNA Sequencer using the Big Dye Terminator Cycle Ready Reaction Kit with Amplitaq DNA polymerase according to the manufacturer's instructions.

Amplification of the DM64 cDNA by PCR with specific primers

The *D. marsupialis* library was used as a template for PCR amplification of the DM64 cDNA. The forward primer DM130F ($5'$ -ttgacctgtaccaggaagg- $3'$) corresponding to the internal amino acid sequence FDLYQE(153–158) of DM64 was used together with *NotI* oligo(dT) reverse primer, which anneals to the poly(A) tail, in the PCR amplification. The PCR was prepared using $1 \mu\text{L}$ of cDNA library solution and 20 pmol of each primer per reaction. The amplification was carried out using a PTC-100 thermal cycler (M.J. Research, USA) according to the following program: $92 \text{ }^\circ\text{C}$ for 5 min followed by 35 cycles ($92 \text{ }^\circ\text{C}$ for 30 s, $45 \text{ }^\circ\text{C}$ for 30 s and $72 \text{ }^\circ\text{C}$ for 3 min) and a further extension step at $72 \text{ }^\circ\text{C}$ for 7 min. The PCR products were electrophoresed in 1% (w/v) agarose gels and the excised fragment was subcloned in pGEM-T-easy vector (Promega). *E. coli* DH5 α cells were used for transformation and plated on 2YT agarose plates containing $100 \mu\text{g} \cdot \text{mL}^{-1}$ ampicillin [34]. Plasmidial DNA was prepared from individual clones using In Concert Plasmid Purification System (Life Technologies), digested with *NotI* and analyzed on a 1% (w/v) agarose gel. Two clones containing the expected size inserts were sequenced using plasmid specific primers (M13F-cccagtcac-gacgttgaataaacg- and M13R-agcggataacaatttcacacagg) (Life Technologies) in both directions. To amplify the upstream region of DM64 cDNA, including the N-terminus, a specific reverse primer DML250R ($5'$ -cagcttgaattccagccag- $3'$) was synthesized based on the nucleotide sequence already obtained. The upstream PCR was prepared with the reverse primer DML250R and the forward primer T7 ($5'$ -taatacagactcactataggg- $3'$), which anneals to the T7 promoter located in the pGEM11Zf+ plasmid. Amplification was carried under the previously described conditions. Based on the sequences obtained, two new primers were synthesized, DML370F ($5'$ -tgccaacatcctgagctacg- $3'$) and DM60F ($5'$ -gagctccagctgtggaaag- $3'$), to complete the sequencing by primer-walking. The complete sequence of DM64 was determined for both strands. Sequence analysis was performed by using the VECTOR NTI SUIT software

(Informax). The cDNA sequence obtained, as well as its deduced amino acid sequence, was compared with sequences in the GenBank database using BLAST Search Program (NCBI, Bethesda, MD).

Myotoxicity *in vivo*

Myotoxicity was analyzed by quantification of plasma creatine kinase (CK) activity using the Sigma n°47–10 kit. Groups of four Swiss–Webster mice (18–20 g) received intramuscular injections (0.1 mL) in the gastrocnemius muscle of myotoxins I (50 µg) or II (70 µg) from *B. asper* mixed with increasing amounts of DM64. Toxins alone were used as positive controls whereas NaCl/P_i or DM64 were injected as negative controls. After 3 h, blood was collected from the tail into heparinized capillary tubes for CK determination. Activity was expressed as U·L⁻¹ (1 unit defined as the amount of enzyme, which produces one µmol of NADH min⁻¹, at 30 °C) [22].

Cytotoxicity *in vitro*

Cytotoxicity was assayed *in vitro* using C2C12 skeletal muscle cells, as described previously [35]. *B. asper* myotoxins I or II (15 µg·150 µL⁻¹) alone or mixed with DM64 at different molar ratios were diluted in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% (v/v) fetal bovine serum and then added to cell cultures growing in 96-well plates. After 3 h incubation at 37 °C, 100 µL aliquots of the supernatant were taken for lactate dehydrogenase (LDH) determination, using Sigma n°500 kit. Controls of 0% and 100% cytotoxicity consisted of medium and 0.1% (v/v) Triton X-100 lysate, respectively.

Complex formation

Complex formation between myotoxin I or II and DM64 was analyzed by native PAGE. Myotoxin I (6.6 µg) or II (3.3 µg) was incubated with DM64 (7.5 µg) and then analyzed on 12% homogeneous gel, stained with Coomassie Blue R-250. Myotoxins and DM64 were used as controls.

Phospholipase A₂ activity

PLA₂ activity was assayed by incubating 0.1 mL of myotoxin I (20 µg) and increasing amounts of DM64 with 1 mL of an egg yolk suspension diluted 1 : 5 with 0.1 M Tris/HCl, pH 8.5, 0.01 M CaCl₂, containing 1% (v/v) Triton X-100. Toxin was used as a positive control whereas NaCl/P_i or DM64 were applied as negative controls. After 20 min at 37 °C, free fatty acids were extracted and titrated according to the method of Dole [36].

Anticoagulant activity

Anticoagulant activity was determined using platelet-poor sheep plasma according to Gutiérrez *et al.* [37]. In brief, myotoxin I (2 µg) and DM64 were incubated at different molar ratios and mixed with 0.5 mL of plasma, for 10 min, at 37 °C. Then, 0.1 mL of 0.25 M CaCl₂ was added to each tube and clotting times were recorded. NaCl/P_i, myotoxin and DM64 were used as controls.

Intracerebroventricular lethality

Groups of four Swiss–Webster mice (16–18 g) received a 10-µL intracerebroventricular injection of myotoxin I (2 µg) mixed with DM64, at different molar ratios [37]. Control groups received identical injections of NaCl/P_i or DM64. After 24 h, the number of dead animals in each group was recorded.

Antihemorrhagic activity

The activity of DM64 against the hemorrhage induced by *B. jararaca* or *B. asper* venoms was tested on rats as previously described [38]. Briefly, animals were injected with a mixture of two minimum hemorrhagic doses of each venom (Bav = 40 µg; Bbv = 42 µg) with increasing amounts of DM64. Venoms or DM64 were used as positive and negative controls, respectively. Hemorrhagic spots were measured after 24 h.

Anti-fibrinogenolytic activity

DM64 was assayed against isolated snake venom metalloproteinases (1 µg of jararhagin or bothrolysin from *B. jararaca* venom) using fibrinogen as substrate [39]. Bovine fibrinogen, prepared as a 5 mg·mL⁻¹ solution in 0.02 M Tris/HCl, pH 7.4, 0.02 M CaCl₂, 0.15 M NaCl, was mixed with the enzymes (10 : 1, w/w) previously incubated for 10 min, at 37 °C, with different amounts of DM64. After hydrolysis for 10 min, SDS/PAGE sample buffer containing β-mercaptoethanol was added, the samples were boiled for 5 min and analyzed by SDS/PAGE. The enzymes were used as positive controls. Total snake venom metalloproteinase (SVMP) inhibition was achieved by adding either 10 µmol of EDTA or an equimolecular amount of DM43 to the enzymes.

Statistical analysis

Results represent mean ± SEM (*n* ≥ 4). Data were statistically evaluated by Analysis of Variance (ANOVA), followed by Newman-Keuls-Student's test. *P*-values of 0.05 or less were considered significant.

RESULTS

Purification procedures

D. marsupialis whole serum was fractionated by ion-exchange chromatography (Fig. 1A) and the heterogeneous DM64 was obtained as the ascending portion of the main acidic peak. This sample was further purified by affinity chromatography (Fig. 1B) and homogeneous DM64 was obtained. SDS/PAGE profiles under reducing conditions of DM64 fractions from each purification step are shown in Fig. 2A. From 2.5 g of serum proteins, 8 mg of homogeneous DM64 were obtained.

Physicochemical and chemical characterization

DM64 has a molecular mass of 63 659 Da by MALDI-TOF MS. SDS/PAGE, under reducing conditions, showed a molecular mass of 66.5 kDa. The molecular

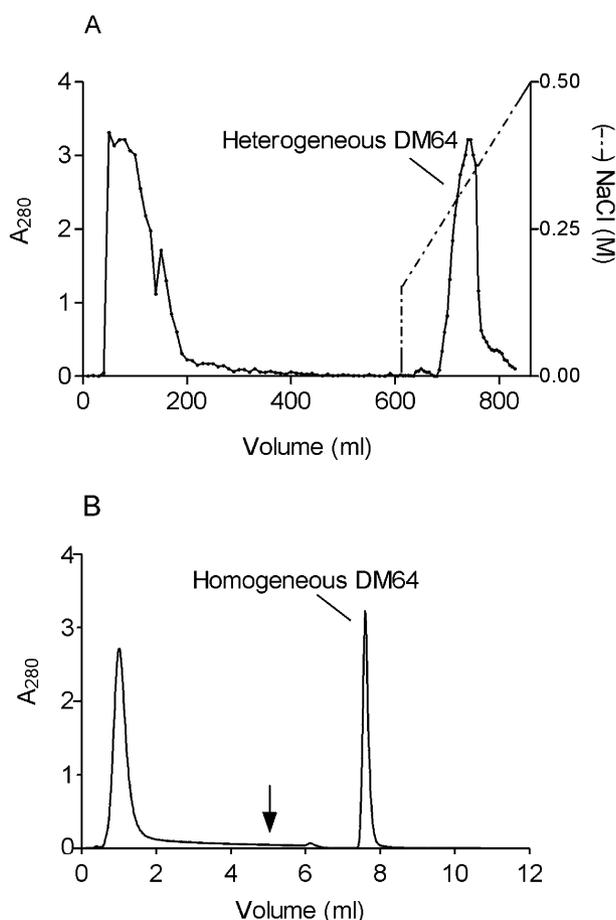


Fig. 1. Purification of DM64. *D. marsupialis* serum was chromatographed on a DEAE-Sephacel column (A) eluted initially with sodium acetate 0.01 M, pH 3.7, followed by a linear gradient from 0.15 to 0.5 M NaCl in the same buffer, at a flow rate of 0.5 mL·min⁻¹. The heterogeneous DM64 fraction was further chromatographed on a Hitrap® NHS-activated affinity column coupled with myotoxin I from *B. asper* (B) equilibrated with 0.02 M sodium phosphate, pH 7.0. The bound fraction was eluted with 0.1 M glycine/HCl, pH 2.7, at a flow rate of 1 mL·min⁻¹.

mass of native DM64 was determined by gel filtration chromatography on Sephacryl S-200 (110 kDa) and on Superdex 200 (86 kDa). Size exclusion chromatography on Superdex 200 in the presence of guanidine-HCl yielded 63 kDa. Chemical cleavage of the DM64 glycan moiety with TFMS reduced its molecular mass by 15%, as determined by SDS/PAGE (Table 1). DM64 was electro-focused between pH 3 and 10 and a major band corresponding to an isoelectric point of 4.5 was observed (Fig. 2B).

Molecular cloning and sequence analysis

The purified protein as well as internal peptides generated after cleavage with CNBr or Lys-C endoproteinase when subjected to Edman sequencing (Fig. 3) showed structural homology to DM43 and to oprin, two SVMP inhibitors previously isolated from *D. marsupialis* and *Didelphis virginiana* serum, respectively [24,40].

As evidenced by the sequence alignment (Fig. 4), the specific primer DM130F was designed based on a region of highly conserved amino acid sequence FDLYQE(153–158), using the corresponding nucleotide sequence of the partial characterized oprin cDNA [40]. The cDNA library prepared from *D. marsupialis* liver and screened by PCR with primers DM130F and *NotI* oligo(dT), resulted in the amplification of a DNA fragment of approximately 1200 bp. This fragment was cloned and two clones were confirmed as positive by restriction analysis. Both were completely sequenced. Using the oligonucleotides DML250R and T7 and the cDNA library as template, the nucleotide sequence was extended by PCR to obtain the N-terminal sequence, signal peptide and the 5'UTR region. The complete DM64 cDNA sequence was obtained by superposing all sequenced fragments. The nucleotide and predicted amino acid sequences, including the DM64 signal peptide, are shown in Fig. 3. The start codon ATG is at nucleotide position 38 and the stop codon TGA was localized at nucleotide 1550. The polyadenylation signal (ATAAA) was observed 15 nucleotides upstream from the poly(A) tail. The N-terminal and three internal peptide sequences generated by Edman chemistry (underlined in Fig. 3) confirmed the cDNA as the genuine coding sequence for DM64. No discrepancy was found between DNA and protein sequencing data. The complete cDNA includes both the 5'- and 3'-UTR.

The deduced protein sequence was searched against the GenBank using BLASTP v. 2.0 software revealing that DM64 has the same high similarity (78%) with DM43 and oprin. In addition, 50% similarity was found with human α_1 B-glycoprotein, a plasma protein of unknown function and a member of the immunoglobulin supergene family [41] (Fig. 4). Each domain of these proteins possesses two cysteine residues at conserved positions (grey boxed in Fig. 4). DM64 also presented four putative N-glycosylation sites (black boxed in Fig. 3), three of them aligning to the same DM43 sites (clear boxed in Fig. 4). A gap of four amino acids beginning after residue 242 of DM64 is also present in human α_1 B-glycoprotein. Such gap was not found on the third domain of DM43.

Inhibitory properties

Myotoxicity induced by *B. asper* myotoxins I and II was almost completely inhibited when a twofold molar excess of DM64 was used (Fig. 5), whereas total inhibition of their cytotoxic activity, as measured by LDH release, was obtained by DM64 at an equimolar ratio (Fig. 6). DM64 alone was devoid of myotoxicity and cytotoxicity in these experimental systems. DM64 did not inhibit enzymatic, lethal and anticoagulant activities of myotoxin I, even when a twofold molar excess of the inhibitor was used (not shown). Myotoxin II was not tested, since it is devoid of these activities. DM64 was also ineffective in the inhibition of *B. asper* or *B. jararaca* venom-induced hemorrhage (not shown). In agreement with this result, DM64 did not inhibit the fibrinolytic activity of the SVMPs jararhagin (Fig. 7A) or bothrolysin (Fig. 7B).

Complex formation

Myotoxins and DM64 were mixed and submitted to electrophoresis under native conditions. A new band stained

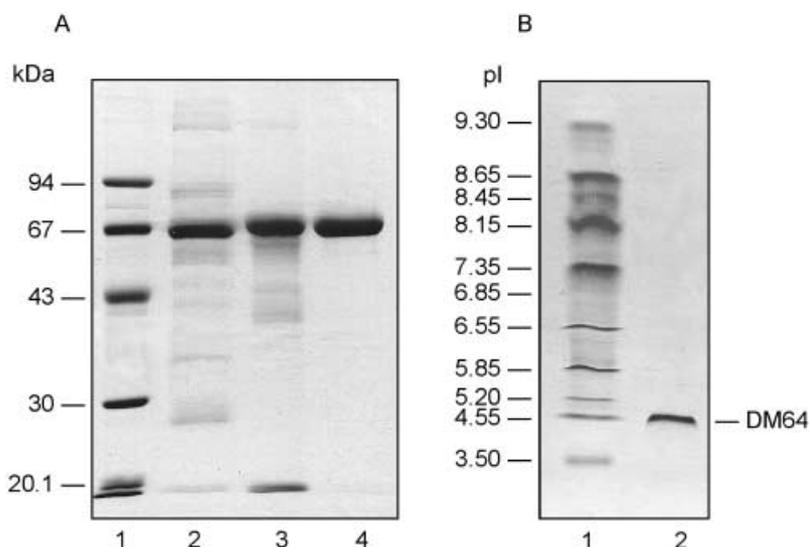


Fig. 2. SDS/PAGE of DM64 chromatographic fractions (A) and determination of isoelectric point (B). (A) Lane 1, molecular mass markers; lane 2, *D. marsupialis* serum (12 µg); lane 3, heterogeneous DM64 from DEAE-Sephacel (6 µg); lane 4, homogeneous DM64 from affinity chromatography (6 µg). Samples were run in the presence of β-mercaptoethanol and the gel was Coomassie Blue stained. (B) DM64 was electrofocused between pH 3 and 10 on a thin-layer polyacrylamide gel. Lane 1, pI calibration standards; lane 2, DM64 (3 µg).

Table 1. Molecular masses of DM64 determined by different methods. ND, not determined.

	SDS/PAGE	MS	Superdex 200	Sephacryl S-200
DM64	66.5 kDa	63 659 Da	86 kDa	110 kDa
DM64 in guanidine-HCl	ND	ND	63 kDa	ND
DM64 (deglycosylated)	56.3 kDa	ND	ND	ND

by Coomassie Blue was visualized, suggesting complex formation between the toxins and the inhibitor. The noncomplexed myotoxins did not enter the gel because of their basic nature (Fig. 8).

DISCUSSION

Animal PLIs described to date have been isolated only from snake plasma and present several common characteristics: they are oligomeric acidic glycoproteins formed by three to six (non)identical subunits linked by noncovalent bonds. Their native molecular masses vary from 75 to 180 kDa and the subunits range from 20 to 50 kDa [12]. At least three PLIs have been shown to exert myotoxin inhibitory properties: BaMIP isolated from *B. asper* [15] and CgMIP-I and CgMIP-II from *C. godmani* [16].

This is the first report of a myotoxin inhibitor isolated from the serum of a mammal. DM64 is an acidic glycoprotein with an isoelectric point of 4.5, comprising 15% carbohydrate. Its molecular mass determined by MALDI-TOF MS was 63 659 Da, in agreement with the value of 63 kDa obtained by gel filtration in guanidine-HCl and 66.5 kDa by SDS/PAGE under reducing conditions. The slightly higher value given by SDS/PAGE is probably consequence of the glycosylated nature of DM64 [42]. The molecular mass of native DM64 was also analyzed using different gel filtration matrices. Upon chromatography on a Sephacryl S-200 column, a value of 110 kDa was obtained,

suggesting that native DM64 exists as a dimer. It also suggests an interaction between the native protein molecule and the Superdex matrix, which would artificially increase its elution volume and decrease its apparent molecular mass to 86 kDa. Similar results were obtained for DM43 and BJ46a, SVMP inhibitors isolated from *D. marsupialis* [24] and *B. jararaca* [43] sera, respectively, both of which are homodimeric proteins in native conditions.

The precise mode of action of class II PLA₂ myotoxins remains elusive. However, it seems clear that the initial target of these toxins is the skeletal muscle sarcolemma. Typically, upon experimental intramuscular injection, these toxins induce the formation of 'delta lesions' followed by hypercontraction of myofilaments due to increased intracellular levels of calcium ions [5,44]. Despite the fact that myotoxic PLA₂s affect a variety of cell types in culture [45], muscle cells show the highest susceptibility [35], indicating the existence of specific targets in muscle cell plasma membrane. The acceptor site could be either a negatively charged phospholipid domain [46] or a protein such as the PLA₂ M-type receptor [47]. In both cases, electrostatic interactions between cationic residues on the surface of the myotoxin and negatively charged groups in the membrane seem to be involved. After this initial binding, myotoxins penetrate the bilayer by a hydrophobic interaction mediated by a cytotoxic region of the molecule, different from the catalytic site, and which combines hydrophobic amino acid residues flanked by cationic residues [44]. In the case of

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1·  AAGACCCTCTGCCTCCAGTCAGGAGAGAGAAGTAGCCATGGCTGCCCCCTTTTATTCTTCT
      M A A P F I L L ·8
61·  ATCTTTCTAGGACTTTGGCTGGACCCCAAGGACAGAGGTGGATGATATATGGCTATGGA
      F F L G L W L D P K A E V D A L L A M E ·28
121· AACAACTCCTCGCTCTGGATCGAGACCGAATCCCATCCACTCCTTGGACCAATGTGAC
      T T P R L W I E T E S P S T P W T V T ·48
181· TCTCCAGTGTGTGGCAACCAATACCGAGGCCCTGAGCTTCCAGCTGTGGAAAGACGGGGA
      L Q C V A T N T E A L S F Q L W K D G E ·68
241· GCTCCTCTCTACTCTCCCTCCATGGGCCCTCGTGGCAAGTTCCTCGTGGGGCCGGTGAC
      L L S T L P P M G L V G K F S L G P V T ·88
301· AGACGACAACAGGGGCTTTATCGTTGCCGGATACTGATGTTTGAAAACACCTGGACCTC
      D D N R G L Y R C R I L M F E N T W T S ·108
361· CCCGAGTGAACAGTGGAGGTGACAGGCAAGAGCCTCTCCCTGCCCCCTTGCTACGGGC
      P S E P V E V T G K E P L P A P L L R A ·128
421· TGATCCTGGACCTTGGATCCTCCATGGCCCTGGAACCAAGCTGCATGCCAAGGGGTGCT
      D P G P L W I L H G L E T K L H C Q G V L ·148
481· TCTGGGCATGATTTTGGACCTGTACCAGGAAGGAGAGCAGGAGCCCGTGAGGAGCTCCA
      L G M I F D L Y Q E G E Q E P V R S S H ·168
541· CACACAGGCACAGAACCACCTTCATTGTCAACAATACCGGGAACACAGCTGCCTCTA
      T P G T E A T F I V N T G Y S C L Y ·188
601· CCGAGCACCTGGCCAGCTTCCAGTGTGAATTGAGCACCAGTGGAGCCATACAGCTGT
      R A P A P A S S V N S A P S E T I H V V ·208
661· GATACCAGACTTACTCCAAAGCCTGACTTCCATATTTACGACAACCAAGTCATCAGGCC
      I P D L L P K P D F H I V D N Q V I R P ·228
721· TGGAGACTCCGTGACATTTGGCTGCTGGGGGAGATTCTCTGGCCTGGAATCAAGCTGT
      G D S V T T F G C W G R F S G L E F K L F ·248
781· CAAAGCCGACAGGAGGTGTTGTACCAAGCAGTCTTCAAAGGACCCCAAGCACATATA
      K D G Q E V F V P K Q S S K D P K H I Y ·268
841· TTTCGAACTGACAGCCCTGGGCCCTGAGGATGGGGCAAGTACAGCTGCAGGTACCGCTT
      F E L T A L G P E D G G K Y S C R Y R F ·288
901· TAGGAATGGCCGCCAATCTGGTCAGAAGACAGTAAGCAACTGGAGCTGGTCTGACTAC
      R N G P P I W S E D S K Q L E L V L T T ·308
961· AGAGACATTAGCCAAGCCTTCCCTGTCTGTGAGCCCCAGGAGACTGTATTTCTCGAGG
      E T L A K P S L S V E P Q E T V I S R G ·328
1021· AACCAAGGTGACCATGCGTTGCCAGGGGGCCAGCCCAATGTGAAATTCGTCTGCTGAA
      T K V T M R C Q G A Q P N V K F V L L K ·348
1081· GAAAGGCTCACCTGGGCACACGTTGGTGTGAGCTCTCCCGAGTCCCATTTGACTTTGT
      K G S P G H T L V L S S P E S H V D F V ·368
1141· COTGCCAAACATCCTGAGCTACGACTGGCAATTTACAGTGCCTTTATGTTCAAACAGA
      L P N I L S Y D T G F S C L Y Q T E ·388
1201· GGCCCCGTTTGTGCTCCCAAAGGAGTGAGGATGTGGAGATCCGAGTGGAAAGCCCTTCT
      A P F A G S Q R S E D V E I R V E G L L ·408
1261· CCCCAAGCCACGCTGCATCCTGTGCACCCCTGTTGTGGCCCCGGGAGGGATGCAATCCT
      P K P T L H P V H P V V A P G R D A I L ·428
1321· GCACTGTTCCAGGAAAGATCCCGAATGCCACTTCCAGCTCTCAAAGATGGAGAATGA
      H C S G K I P N A H F Q L F K D G E H E ·448
1381· AGAGCTGGAAGTCACTGTTTGGCCATTGATGACCATGCTGTAACCTCTCTTGAAGAA
      E L E V S V L P I D D H A V N F L L K N ·468
1441· TATCAATCGCCAGCAGGGGGCAAGTACAGGTGCGCCTATACCACAGGGAGGATCCCAT
      I N R Q Q G G K Y R C R Y T T R E D P I ·488
1501· CTTGGAGTCAGAGATGAGTACCCCTGCAGAGCTTCAAGTACAGGCGGCTGAGCCGAGGC
      L E S E M S D P A E L Q V T G Q * ·504
1561· CCCTCCTTCCCACCAAGTCTGCCAAGCAGCGCGCTGCGCGGCGGCCGCCGAACAGC
1621· CCGCTGGCTCCGGCTCCCCATAAAGCTCGCCGTGAAGCCAAAAAATAAAAAAAAAA
1681· AAAAAAAAAA

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Fig. 3. Complete cDNA sequence of DM64 and its deduced protein. Sequence in bold corresponds to the signal peptide. Underlined residues were confirmed by Edman sequencing of DM64 and of CNBr and endoproteinase Lys-C derived peptides. The polyadenylation signal is doubly underlined. Solid arrow corresponds to primers designed to amplify DM64 cDNA and dotted arrows indicate the primers designed to complete the sequence by primer walking. The four putative N-linked glycosylation sites are black boxed. The start codon ATG and the stop codon TGA are grey boxed.

catalytically active PLA₂, the membrane disorganization seems to be potentiated by enzymatic degradation of phospholipids [7,44].

The capacity of DM64 to inhibit the myotoxicity induced by myotoxins I (Asp49) and II (Lys49) from *B. asper* venom was analyzed *in vivo* and *in vitro*. DM64 effectively neutralized the myotoxic and cytotoxic effects of both myotoxins. Interestingly, DM64 did not inhibit the PLA₂ activity of myotoxin I nor its intracerebroventricular lethality and its anticoagulant effect, activities that depend on the enzymatic activity of this protein [44]. These results confirm that the myotoxicity induced by mt-I is not dependent on its catalytic activity. The dissociation between these two activities was previously demonstrated using monoclonal antibodies, which were able to neutralize myotoxicity without inhibiting mt-I enzymatic activity [48]. In addition, it was observed that chelation of calcium ions completely inhibited the toxins' enzymatic activity, although residual myotoxicity was still observed. Furthermore, the existence of Lys49 PLA₂ structural variants displaying myotoxic

activity suggests that enzymatic activity is not an essential requirement to induce muscle damage [44,49]. Native PAGE and affinity chromatography indicate that DM64 forms noncovalent soluble complexes with myotoxins I and II. As mentioned above, in the case of mt-I, the enzymatic activity was not affected. Furthermore, one can speculate that DM64 binds to the myotoxins through a myotoxic/cytolytic site distinct from the catalytic site, as already described for the inhibition of myotoxicity by heparin [44]. At least in the case of *B. asper* mt-II [50] and of a Lys49 PLA₂ from *Agkistrodon piscivorus piscivorus* [51], a cytolytic heparin-binding domain has been located on the C-terminal region of the molecule.

In contrast to the antimyotoxic proteins described so far, DM64 is structurally related to DM43 [24] and to α_1 B-glycoprotein, a single chain human serum protein with unknown function, and a member of the Ig supergene family [41]. It has been proposed that Ig-like proteins arose by duplication of a primordial gene coding for about 95 amino acid residues [52]. Recently, it was reported that



Fig. 4. Alignment of the deduced DM64 amino acid sequence with other similar proteins. Sequences were obtained from GeneBank data base and are listed as follows: DM43 from *D. marsupialis* (accession no. P82957), oprin partial sequence from *D. virginiana* (accession no. AAA30970) and human α_1 B-glycoprotein (accession no. AAL07469). The half-cysteine residues that form the internal disulfide bridge of each domain are shown in boxes (grey). Three of the four putative N-glycosylation sites that align to the same DM43 sites are clear boxed. Also shown in boxes (black) are the degenerated WSXWS sequences. The conserved aromatic residues phenylalanine and tyrosine typical of the Ig-fold are bold in each domain.

DM43 has three Ig-like domains [24], while α_1 B-glycoprotein is a five-Ig-like domain protein of 63 kDa [41]. Considering the results on amino acid sequence and molecular mass of DM64, DM43 and α_1 B-glycoprotein, it

can be suggested that DM64 contains five Ig-like domains. A comparison of the first three domains of DM64 and α_1 B-glycoprotein shows that they are homologous to the three DM43 domains [24]. Each of these domains in the three proteins possesses typical signatures of the Ig-fold, namely: two cysteine residues forming a disulfide bridge (grey boxed in Fig. 4) and the aromatic residues phenylalanine and tyrosine (bold in Fig. 4) at conserved positions. The two extra domains present in DM64 possess these same signatures, except that in the fourth domain F380 replaces tyrosine. Also, DM64 shows in its sequence the presence of degenerated WSXWS boxes (black boxed in Fig. 4) [53], which are related to those found in DM43 first three domains and are present in the inhibitory receptors of

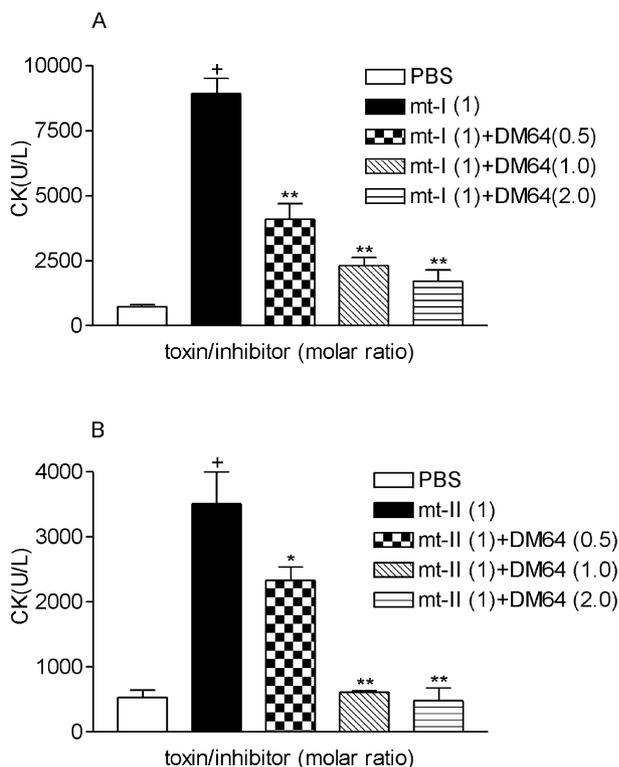


Fig. 5. Inhibition of *in vivo* myotoxicity of myotoxins I or II by DM64. Groups of four mice were injected intramuscularly with 50 μ g mt-I (A) or 70 μ g mt-II (B) alone or mixed with DM64 at different molar ratios. After 3 h, blood was collected from the tail and creatine kinase activity was determined. $^+P < 0.0001$ when compared to NaCl/P_i, $^{**}P < 0.001$ and $^*P < 0.05$ when compared to toxins.

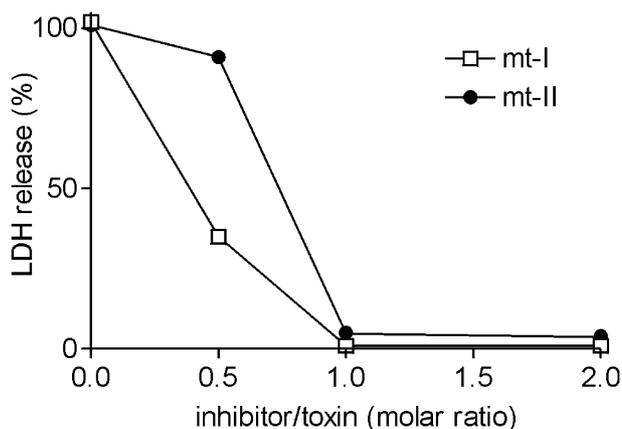


Fig. 6. Inhibition of *in vitro* cytotoxicity of myotoxins I or II by DM64. Cytotoxicity was analyzed *in vitro* using C2C12 skeletal muscle cells. Toxins (15 μ g) alone or mixed with increasing amounts of DM64 were incubated with the cells for 3 h at 37 °C. After incubation, the concentration of LDH released by damaged cells was determined in 100 μ L aliquots of the culture supernatants. Full cytotoxic activity (100%) was defined as the amount of LDH released upon lysis of monolayer controls by addition of 0.1% (v/v) Triton X-100.

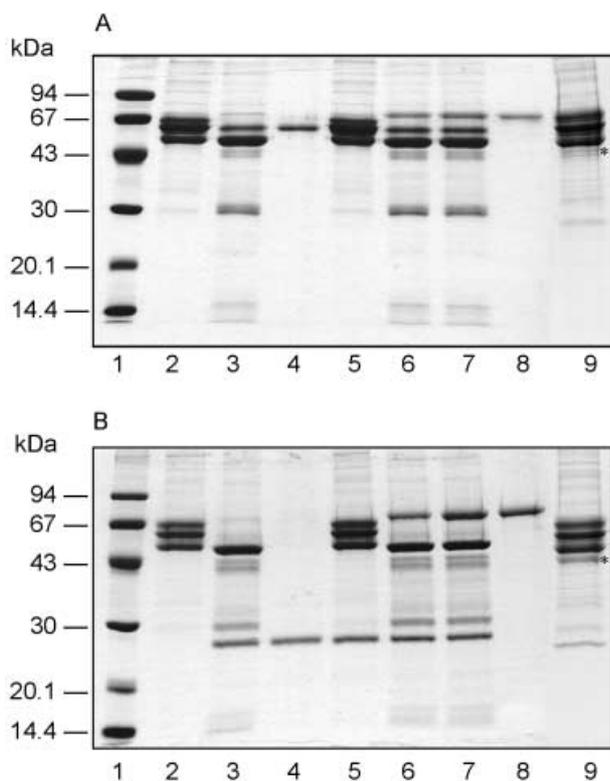


Fig. 7. Hydrolysis of fibrinogen by the SVMPs jararhagin (A) or bothrolysin (B) from *B. jararaca* venom and its inhibition by DMs. Lane 1, molecular mass markers; lane 2, fibrinogen control; lane 3, fibrinogen + SVMP; lane 4, SVMP; lane 5, fibrinogen + (SVMP + EDTA); lane 6, fibrinogen + (SVMP + DM64, 1 : 1, mol:mol); lane 7, fibrinogen + (SVMP + DM64, 1 : 2, mol:mol); lane 8, DM64; lane 9, fibrinogen + (SVMP + DM43, 1 : 1, mol:mol). The position of DM43 on the gel is indicated (*). Samples were analyzed on 12% SDS/PAGE and stained with Coomassie Blue.

natural killer cells [54]. The last two C-terminal domains of DM64 also have regions that are candidates for other tryptophan boxes. The five degenerate boxes that have serine/threonine residues in the second and fifth positions as its main characteristic are found in positions WTSPS(106–110), NSAPS(198–202), WSEDS(295–299), GSQRS(393–397) and ESEMS(490–494). Therefore, DM64 should be considered a member of the immunoglobulin supergene family, which already comprises several proteins involved in the vertebrate immune response, such as antibodies, T-cell antigen receptor and histocompatibility antigens [52]. In spite of the structural similarities between DM43 and DM64, the latter does not present any antihemorrhagic activity against *B. jararaca* or *B. asper* venoms. In contrast to DM43 [20,24], DM64 did not inhibit the fibrinogenolytic activity of bothrolysin (P-I) or jararhagin (P-III) nor formed a complex with them (not shown).

After comparing DM43 with other members of the immunoglobulin supergene family, loops in the region between the second and third domains were predicted to form the metalloproteinase-binding site [24]. A remarkable difference between the sequences of DM64 and DM43 is the presence of a gap of four amino acids in DM64, when compared to DM43. Since this gap is localized in the third

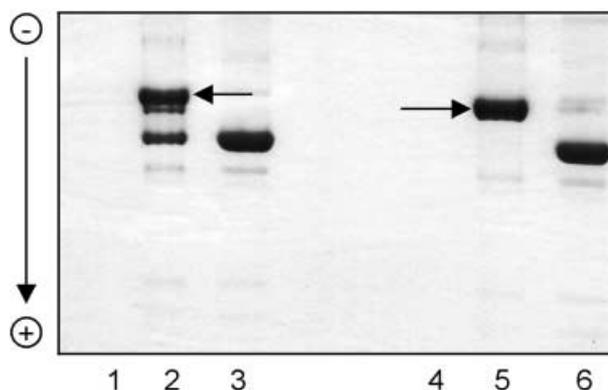


Fig. 8. Complex formation between DM64 and myotoxins I or II. Samples were incubated for 30 min at 37 °C and analyzed for complex formation on 12% native PAGE. Lane 1, myotoxin I (6.6 µg); lane 2, DM64 + myotoxin I; lane 3, DM64 (7.5 µg); lane 4, myotoxin II (3.3 µg); lane 5, DM64 + myotoxin II; lane 6, DM64 (7.5 µg). The gel was Coomassie Blue stained. Black arrows indicate the complex formed.

domain of DM64, in one of the loops previously proposed as one of the regions responsible for ligand binding in DM43 (residues 216–224) [24], it is likely that this loss in DM64 affected its interaction with metalloproteinases, inducing the loss of its antihemorrhagic activity. Moreover, the most striking difference between DM43 and DM64 is the presence of two extra domains at the C-terminal side. This may suggest that the myotoxin binding region is located in loops of these extra Ig-like domains, indicating that the shift from an antihemorrhagic to an antimyotoxic molecule could be the result of a combination of these two features, presence of the gap in the third domain and the two extra domains at the C-terminal in the DM64 molecule.

Analysis of DM64 structural and biological properties showed that at least one of its physiological functions is to afford circulating protection against foreign toxins, therefore indicating that DM64 performs functions of the innate immune system. It is remarkable that two proteins with Ig-like structure, DM43 and DM64, have two completely different activities, the former being a metalloproteinase inhibitor and the latter an antimyotoxic protein. Both of them play different, yet complementary, roles in the resistance of opossum to snake venoms.

In conclusion, DM64 is a novel PLA₂ myotoxin inhibitor with Ig-like structure and without PLA₂ inhibitory activity, which is likely to contribute to the resistance of *D. marsupialis* against snake venoms.

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