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CLONING OF A NOVEL ACIDIC PHOSPHOLIPASE A_2 FROM THE VENOM GLAND OF *Crotalus durissus cascavella* (BRAZILIAN NORTHEASTERN RATTLESNAKE)

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ABSTRACT: The phospholipase A_2 superfamily encompasses 15 groups that are classified into: secreted PLA_2 ($sPLA_2$); cytosolic PLA_2 ($cPLA_2$); Ca^{2+}-independent intracellular PLA_2 ($iPLA_2$); platelet-activating factor acetylhydrolase (PAF-AH); and lysosomal PLA_2. Currently, approximately 700 PLA_2 sequences are known, of which 200 are obtained from the venom gland of Crotalinae snakes. However, thus far, little information is available on cloning, purification and structural characterization of PLA_2 from *Crotalus durisssus cascavela* venom gland. In the present work, we report the molecular cloning of a novel $svPLA_2$ from *C. d. cascavella* (*Cdc*), a predominant rattlesnake subspecies in northeastern Brazil. The *Cdc* $svPLA_2$ cDNA precursor is 689 nucleotides long and encodes a protein of 138 amino acid residues, with a calculated molecular mass of approximately 13,847 Da and an estimated isoelectric point of 5.14. Phylogenetic analysis of Crotalinae PLA_2 reveals that *Cdc* PLA_2 clustered with other acidic type IIA PLA_2 homologues is also present in the venom of North American rattlesnakes. Hitherto, this study presents a novel PLA_2 cDNA precursor from *C. d. cascavella* and data reported herein will be useful for further steps in $svPLA_2$ purification and analysis.

KEY WORDS: molecular toxinology, *Crotalus durissus cascavella*, snake venom gland, cDNA library, acidic PLA_2.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The superfamily of phospholipase A_2 enzymes is currently subdivided into 15 groups based on their structures, source and localization. Distributed among these groups are the multiple forms of secreted PLA_2s ($sPLA_2$s – groups I, II, III, V, IX, X, XI, XII, XIII and XIV), cytosolic PLA_2s ($cPLA_2$ – group IV), Ca^{2+}-independent intracellular PLA_2s ($iPLA_2$ – group VI), platelet-activating factor acetylhydrolases (PAF-AH – groups VII and VIII) and the lysosomal PLA_2s (group XV) (1).

Secreted PLA_2s are found in fungi, bacteria, plants, marine sponges, cnidarians, mollusks, starfishes, insects, reptiles and mammals (1-4). Essentially, these enzymes catalyze the hydrolysis of different membrane phospholipids at the *sn*-2 position, releasing free fatty acids such as arachidonic acid (AA) – a precursor of bioactive eicosanoids – and lysophospholipids (lyso-PL). Both products represent the first step in generating second messengers that play important physiological and pathological roles. Lyso-PL can be converted into lysophosphatidic acid (LPA), involved in cell proliferation, survival and migration, or into platelet activating factor (PAF), implicated specifically in inflammatory processes (5, 6). Eicosanoids affect body mechanisms including sleep regulation, immune response, inflammation and pain (7).

PLA_2s from Viperidae venoms ($vPLA_2$) belong to the IIsubgroup together with mammalian enzymes isolated from the spleen, mast cells, macrophages, arthritic synovial fluid and serum of patients with inflammatory diseases (8-10). This subgroup is characterized by low-molecular-mass enzymes (~14 kDa), with a rigid three-dimensional structure composed of seven disulfide bridges, whose catalytic mechanism utilizes a His-Asp dyad. These enzymes require a millimolar concentration of Ca^{2+} to exert their enzymatic action and, in contrast to $cPLA_2$s, they have low specifity for arachidonic acid at the *sn*-2 position (11).

Approximately 700 sequences from type II PLA_2s are known and compiled in databases. The diversity of snake venom PLA_2 functions includes: neurotoxicity, cardiotoxicity, myotoxicity, edema, hypotension, hyperalgesia as well as activation and inhibition of platelet aggregation (12-19). The diversity of biological and pharmacological functions of PLA_2 denotes that accelerated or positive Darwinian evolution has occurred and appears to confer a better fitness on the snake venom (10, 20). In fact, the venom PLA_2 subgroup II is further subdivided into two other smaller subgroups, $vPLA_2$s exhibiting enzymatic activity and a predominance of two types of amino acid residues at the catalytic site (position 49) – Asp (D49) and Ser

(S49) – and non-enzymatic $vPLA_2$s (that is, $vPLA_2$ with extremely low enzymatic activity), whose residues, D49 or S49, were replaced not only with Lys (K49), but also Gln 49 (Q49), Ala (A49) and Asn (N-49) (21-26). Furthermore, D49 PLA_2 also includes acidic and basic toxic components that are found in venoms as monomers or homo- and heterodimers (27).

In this work, we report a novel PLA_2 cDNA precursor of *Crotalus durissus cascavella* venom, in which the predicted protein was clustered with acidic members of the type II subfamily of venom PLA_2.

MATERIALS AND METHODS

Specimens of Snake Venom Gland

For the construction of the venom gland cDNA library, a pair of glands was excised from a male adult specimen of *Crotalus durissus cascavella* (2 kg weight and 125 cm length – measured from rostrum to cloaca) captured in Cabaceira, Paraíba state, Brazil, and maintained from 1999 to 2006 in the Laboratory of Venomous Animals and Toxins (LAPTOX), Federal University of Pernambuco, Recife state, Brazil. The snake venom was extracted by standard procedures three days before the surgery for gland excision, with the aim of reaching the maximal level of RNA synthesis. Once surgically removed, the venom glands were kept at –80°C until the procedures for RNA purification and analysis.

Construction of *C. d. cascavella* Snake Venom cDNA Library

A *Crotalus durissus cascavella* venom cDNA library was constructed from 1 µg of total RNA, as follows: frozen venom glands were finely crushed in a mortar with a pestle under liquid nitrogen; then, total RNA was purified using Trizol® reagent (Invitrogen, USA), according to the manufacturer's instructions. The quality and yield of total RNA were verified by the integrity of 28S and 18S rRNA, through denaturing agarose gel electrophoresis using the spectrophotometric ratio 260/280 nm. Poly(A^+)-RNA was purified from total RNA by a complex of oligo(dT)-biotin and streptavidin MagneSphere® paramagnetic particles (PolyATract® system, Promega, USA). Next, mRNA was quantified and employed for cDNA synthesis using the switching mechanism at the 5' end of RNA transcriptiom (SMART) protocol (Creator SMART cDNA Library Construction kit®, BD Biosciences, USA), which preferentially enriches the final library with full-length cDNA.

Cloning of *Cdc* PLA_2 cDNA and Nucleotide Sequencing

The *C. durissus cascavella* venom gland cDNA library was then titered and pools of approximately 10^6 colony forming units (CFU) were used as a template in ten separate homology screening polymerase chain reactions (HR-PCR). Each reaction, in a final volume of 50 μL, in high fidelity PCR buffer (60 mM Tris-SO_4, pH 8.4, 18 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$), consisted of 2.5 U of Platinum® Taq DNA polymerase (Invitrogen Life Technologies, USA), 2 mM $MgCl_2$, 1 mM dNTPs, and 0.2 μM of each forward and reverse primer.

Of the two primers utilized to isolate the *Cdc* PLA_2 cDNA, one (called *Cdc*_PLA_2 sense primer, 5'-TGCACGACTGYTGYTAYGGA-3') anneals to the specific gene sequence, corresponding to the amino acids -FVHDCCYG-, which are conserved in most snake venom PLA_2s, and the other oligonucleotide primer to the plasmid vector (M13 reverse, 5'-AACAGCTATGACCATGTTCA- 3'), which corresponds to the flanking region of insertion in the pDNR-LIB vector.

The cloned full length *Cdc* PLA_2 was automatically sequenced by the dideoxy chain termination method, using the dye-terminator chemistry (DYEnamic ET Dye Terminator® kit, GE Healthcare, USA) and the MegaBACE 750 DNA Analysis System® (GE Healthcare, USA). The PLA_2 gene was *in silico* translated, and both nucleotide and amino acid sequences were compared against a database of genes and proteins, maintained by the NCBI (http://www.ncbi.nlm.nih.gov).

Crotalus durissus cascavella (*Cdc*) PLA_2 Aligment and Phylogenetic Analysis

The deduced amino acid sequence of *C. d. cascavella* PLA_2 cDNA precursor (present study) was compared with the GenBank (http://www.ncbi.nlm.nih.gov) by using the BLAST program (28). This search retrieved 182 protein sequences corresponding to all Crotalinae PLA_2s available in the database. The incomplete and redundant sequences were manually removed from the data set whereas the file with complete sequences was processed for alignment through the multialignment bioinformatic tool ClustalW2, available at the European Bioinformatic Institute website (http://www.ebi.ac.uk). The structural characteristics of the predicted PLA_2 precursor were manually annotated based on data from the literature. Precursors of sequences from Crotalinae PLA_2 toxins which presented higher PLA_2 member scores in comparison with *Cdc* PLA_2 were aligned with MUSCLE 3.6 using groups of amino acids – GA, ST, MVLI, KR, EQDN, FWYH, C and P – to determine the grade of similarity (29).

The evolutionary history was inferred using the neighbor-joining method by analyzing all sequences together, including not only higher score sequences, but also the most dissimilar PLA_2s (30). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are defined as collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (31). The evolutionary distances were computed using the Dayhoff matrix-based method expressed as the number of amino acid substitutions per site (32). All positions containing gaps or missing data were eliminated from the dataset (complete deletion option). There was a total of 85 positions in the final dataset. Phylogenetic analyses were conducted with MEGA4 (33).

RESULTS

A Novel PLA_2 cDNA Precursor of *Crotalus durissus cascavella* Venom Gland

By a homology cloning method, a novel PLA_2 precursor, called Cdc-PLA_2, was retrieved from the venom gland cDNA library of *C. d. cascavella*. As indicated in Figure 1, the Cdc-PLA_2 cDNA precursor is 689 nucleotides long, with an open reading frame (ORF) of 453 nucleotides. The ORF encodes a complete precursor of 138 amino acid residues, including a signal peptide of 16 residues (MRTLWIVAVLLLGVEG). The novel Cdc-PLA_2 cDNA sequence was submitted to GenBank and received the accession number GQ466583.

Comparative Sequence Analysis of the Novel Cdc-PLA_2

The complete amino acid sequence of Cdc-PLA_2 precursor was predicted from a cDNA sequence (Figure 1). Based on this deduced sequence, an isoeletric point of 5.14 and molecular mass of 13,846.81 Da was calculated.

The Cdc-PLA_2 conserved residues involved in Ca^{2+} binding (Tyr28, Gly30, Gly32 and Asp49) and in the catalytic network (His48), characterizing the D-49 group, and maintained conserved sequence domains common to the group IIA PLA_2, including the 14 cysteines responsible for disulfide bond formation.

Phylogenetic analysis of Cdc-PLA_2 and 54 precursors of Crotalinae PLA_2 showed that the maximum grade of parental relationship of Cdc-PLA_2 occurs with acidic PLA_2

from North American snakes (Figure 2). In this case, best similarity values (identities in the range of 60 to 86%) are observed in North American rattlesnakes, for example *Crotalus v. viridis* (86%).

The sequence was aligned with precursors of other Crotalinae PLA_2s, obtained by BLASTp search, which included PLA_2 with amino acid replacement at the position 49, crotapotin from *C. d. terrificus* and the acidic subunit of crotoxin (CA) sequence from *C. d. cascavella* (34). Several residues were highly conserved in the monomeric/homodimeric acidic PLA_2 analyzed in the present work as the N-terminal region (L2XXFE6), Ca^{2+} binding site (Y25GCYCGXGG33), active site (D42RCCFVHDCCYGK54) and C-terminal region (A101AXCFFDN108, Y112, Y117) (Figure 3). On the other hand, the residues A53, D79, S108 and G129 present in heterodimeric toxins (crotoxin A from *C. d. terrificus, C. d. cascavella* and *C. s. scutulatus*) are replaced in Cdc-PLA_2 and in the majority of the mono/homodimeric acid PLA_2s analyzed. Comparative analysis revealed that the hot spot of Cdc-PLA_2 mutations were found at the residues D4, I10, A34, V39, V77, K78, E85, D86, T94, G99, R118 and R128.

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>cDNA:Clone 4.8_ Cdc PLA2

...................................tcctcgccttaccgaacgacgagcg
cagcgagtcagtgagcgaggaagcggccgcataacttcgtatagcatacattatacgaag
ttatcagtcgacggtaccggacatatgcccgggaattcggccattacggccggggggagg
atgaggactctctggatagtggccgtattgctgctgggcgtcgaggggagcctgttggac
 M  R  T  L  W  I  V  A  V  L  L  L  G  V  E  G  S  L  L  D
tttgagatgatgatcattaaagtggcaaagaaaagcggtttgctttggtacagcgcttac
 F  E  M  M  I  I  K  V  A  K  K  S  G  L  L  W  Y  S  A  Y
ggatgctactgcggctgggggggccaaggccgaccacaggttgccactgaccgctgctgc
 G  C  Y  C  G  W  G  G  Q  G  R  P  Q  V  A  T  D  R  C  C
tttgtgcacgactgctgttacggaaaagtgaccgactgcaaccccaaaatggtcagctat
 F  V  H  D  C  C  Y  G  K  V  T  D  C  N  P  K  M  V  S  Y
acctacagcgtgaaaaacggggaaatcatctgcgaagacgacgacccgtgcaagaagcag
 T  Y  S  V  K  N  G  E  I  I  C  E  D  D  D  P  C  K  K  Q
acttgtgagtgtgatggggtcgcggcagtctgcttccgagacaatataccctcatacgac
 T  C  E  C  D  G  V  A  A  V  C  F  R  D  N  I  P  S  Y  D
aagaagtataggcagttcccggccgaaaattgccgggaggaaccagagccatgctaagtc
 K  K  Y  R  Q  F  P  A  E  N  C  R  E  E  P  E  P  C  -

tctgcaggcccgggaaaaacctcaaattacacaattgtagttgtgttactctattattct
gaatgcatactgagtaataaacaggtgccagctttggacttaaaaaaaaaaaaaaaaaaa
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Figure 1. Nucleotide and amino acid sequences of a novel PLA_2 precursor from the venom gland of *C. d. cascavella* (*Cdc*). The Cdc-PLA_2 cDNA precursor (accession number GQ466583) is composed of 689 nucleotides, with an open reading frame (ORF) of 453 nucleotides, which is shown in bold lower-case letters. The ORF encodes a precursor of 138 amino acid residues, shown in a single letter code. The signal peptide of 16 residues is boxed in gray and the residues His48 (H) and Asp49 (D) of catalytic and calcium binding sites, respectively, are underlined in a gray box.

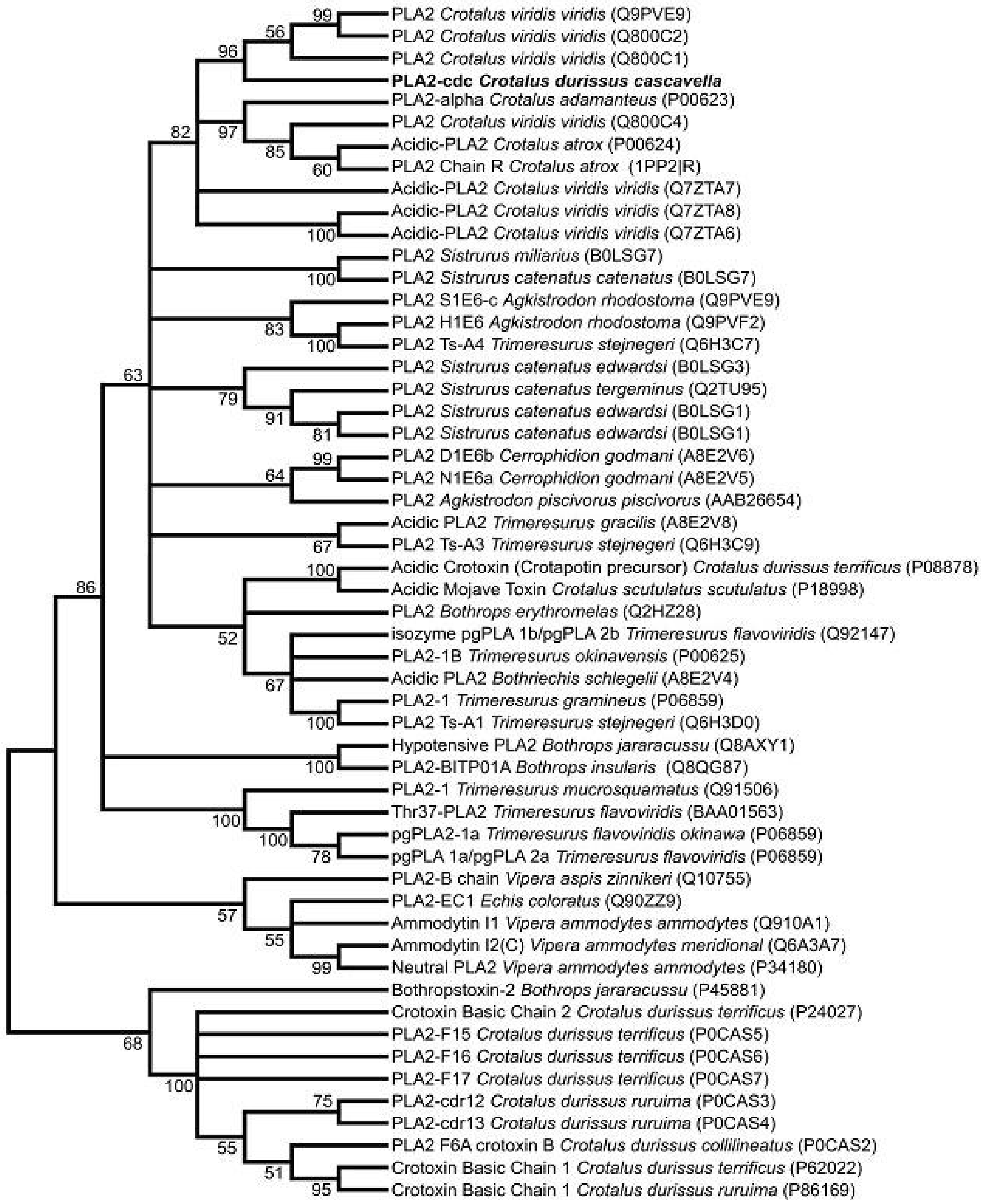

Figure 2. Evolutionary relationship of Cdc-PLA$_2$ and Crotalinae venom PLA$_2$s. The evolutionary history was inferred using the neighbor-joining method. The optimized tree is shown. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (50% cutoff) is presented next to the branches. The tree is drawn to scale, with branch lengths in the same units as those

of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps or missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses are described in the Materials and Methods section. Three main branches are evidenced: one composed of acidic PLA_2, where Cdc-PLA_2 has clustered; the other characterized by a mixture of precursor sequences, encompassing acidic, basic and neutral PLA_2s, as exemplified by the branch of *B. erythromelas* PLA_2; the third is constituted by basic PLA_2, homologous of crotoxin.

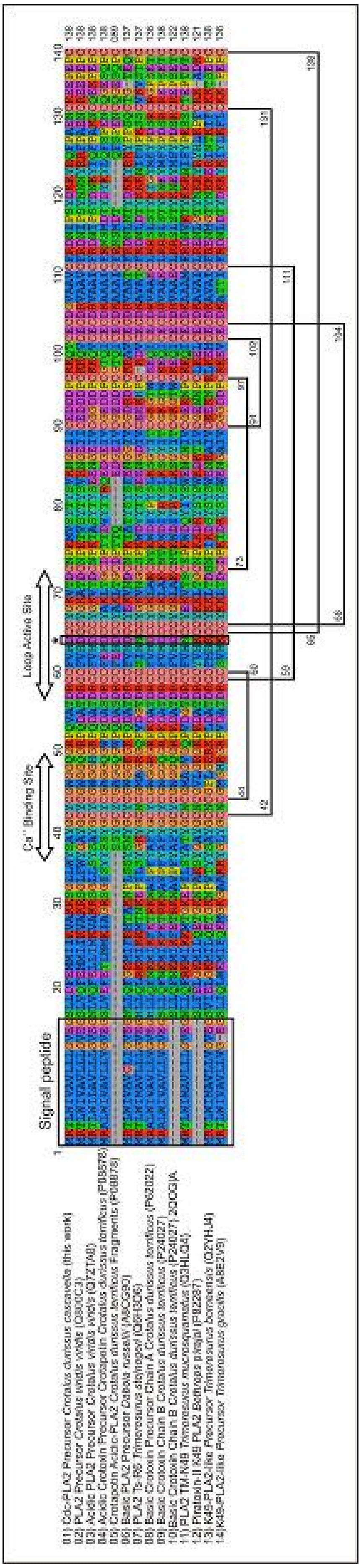

Figure 3. Comparison of amino acid sequence of Cdc PLA_2 precursor with other representative members of PLA_2 subgroups. The deduced amino acid sequence of Cdc PLA_2 precursor was aligned with other acidic PLA_2s, PLA_2 with amino acid replacement at position 49, crotoxin and crotapotin from *C. d. terrificus*. Snake species names are followed by the accession number of each sequence, in parenthesis. Conserved residues appearing in more than 60% of all aligned sequences are colored (60% cutoff), as follows: KR, red; DENQ, blue; ST, green; FHWY, cyan; C, pink; P, magenta; ILMV, yellow; AG, orange. The pattern of S-S bond formation is also indicated, as well as the main functional domains. The alignment was done by the algorithm MUSCLE, as described in the Materials and Methods section.

DISCUSSION

Although studies involving snake venom acidic PLA_2s have increased considerably in the recent years, only a few acidic PLA_2s from Brazilian snake venoms were purified and cloned (18, 21, 35-41). Up to date, nothing was known about the expression of acidic (subgroup II) PLA_2 in the venom gland of *Crotalus durissus cascavella*.

Cdc-PLA_2 possesses high similarity with a subgroup of acidic D49-PLA_2s which is expressed in the venom as monomers and/or as homodimers (42-45). In fact, the ability of an acidic glycosylated and phosphorylated PLA_2s to co-exist in snake venom as monomer and homodimer was recently described by Sun *et al.* (27).

Experimental investigations with native toxins have shown that such group of PLA_2 presents enzymatic activity and capacity of binding calcium ions for maximal catalysis, as seen by the conserved residues His48 and Asp49 in the primary sequences (39, 46-47). These acidic PLA_2s can also induce myotoxicity, platelet aggregation inhibition, hypotension, prostaglandin I2 induction or paw edema (16, 18, 21, 23, 35, 37, 39, 42, 45, 48-50). Some residues associated with antiplatelet (W21, Y113, D114) and edema-forming activities (K78 and D85) are conserved in Cdc-PLA_2 and in some very similar acidic PLA_2 isoforms from *C. v. viridis* venom (43, 50-51). All analyzed acidic PLA_2s presented Glu residue in the position 6, which seems an ancient condition of basic G6 and N6 PLA_2 (21, 52).

Cdc-PLA_2 possesses lower similarity with the other subgroup of acidic D49-PLA_2s (particularly, A chain crotoxin-CA) which can make high stable complexes with basic F24N6 PLA_2 (B chain crotoxin-CB) and increase the toxicity of CB in several folds (52, 53). Except for E124 residue, all amino acids that could be involved in the recognition and binding of a CA with CB (W36, E47, A53, D79, E124 and G129) are replaced in Cdc-PLA_2, what consequently suggests, at a first glance, the impossibility of this toxin to be an A chain crotoxin precursor for heterodimer formation (52). However, this point deserves more attention and further functional and structural analysis.

Acidic IIA phospholipases A_2 present multiple isoforms, generally associated with intra-specific geographic variation, as well as adaptation to prey diversity (43, 44, 54, 55). On the other hand, some PLA_2 clones apparently not translated into venom proteins has been reported, since not-expressing toxin mRNA may be a repository for snake survival under an ever-changing environment (54, 55).

In this work, we report the molecular cloning of an acidic PLA_2 type II from the venom gland of *C. d. cascavella*. Phylogenetic and structural analyses allowed us to make evident that the precursor, retrieved from the *C. d. cascavella* venom gland cDNA library, is a novel member of acidic PLA_2 subgroup. Moreover, a global analysis has shown that the most ancestral member of all PLA_2 precursors in the venom of Crotalinae snakes seems to be related to the crotoxin.

Altogether, the present data will be useful, for example, to drive steps of purification and structural analysis of such flexible and fast evolving snake venom molecule.

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