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Research paper

Isolation and characterization of four medium-size disintegrins from the venoms of Central American viperid snakes of the genera *Atropoides*, *Bothrops*, *Cerrophidion* and *Crotalus*

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ABSTRACT

Four disintegrins were isolated from the venoms of the Central American viperid snakes *Atropoides mexicanus* (atropoimin), *Bothrops asper* (bothrasperin), *Cerrophidion sasai* (sasaimin), and *Crotalus simus* (simusmin). Purifications were performed by reverse-phase HPLC. The four disintegrins have biochemical characteristics, i.e. molecular mass and location of Cys, which allow their classification within the group of medium-size disintegrins. All of them present the canonical RGD sequence, which determines their interaction with integrins in cell membranes. The disintegrins inhibited ADP and collagen-induced human platelet aggregation, with similar IC_{50}s in the nM range. In addition, disintegrins inhibited the adhesion of an endothelial cell line and a melanoma cell line to the extracellular matrix proteins type I collagen, laminin, fibronectin, and vitronectin, albeit showing variable ability to exert this activity. This study expands the inventory of this family of viperid venom proteins, and reports, for the first time, disintegrins from the venoms of species of the genera *Atropoides* and *Cerrophidion*.

1. Introduction

Viperid snake venoms are complex mixtures containing many different proteins, both enzymatic and non-enzymatic [1]. Proteomic analysis of viperid venoms has revealed the presence of a limited number of protein families, with predominance of metalloproteinases (SVMPs), phospholipases A_2 (PLA_2s), and serine proteinases, in addition to other families such as L-amino acid oxidases, C-type lectin/lectin-like proteins, cysteine-rich secretory proteins (CRISPs), disintegrins, and vasoactive peptides, such as bradykinin-potentiating peptides, and other minor components [2].

SVMPs are key components of viperid snake venoms, and participate in the pathogenesis of envenomings owing to their ability to disrupt the microvasculature and induce hemorrhage [3]. SVMPs belong to the M12 family of reprolysins, and have been classified into three classes according to their domain structure: 1) P-I, 20–30 kDa enzymes containing the metalloproteinase domain only; 2) P-II, 30–60 kDa proteins containing a disintegrin domain at the C-terminus; and 3) P-III, 60–100 kDa enzymes comprising an N-terminal metalloproteinase domain, followed by a disintegrin-like domain and a cysteine-rich domain [4]. Some P-III SVMPs present, in addition to the main chain, a smaller subunit constituted by a C-type lectin-like protein, linked to the main chain through disulfide bonds [4]. Various subclasses have been described in P-II and P-III SVMPs on the basis of post-translational cleavage processes and dimerization [4].

Many P-II SVMPs undergo a post-translational cleavage of the disintegrin domain from the main polypeptide chain, with the release of disintegrins [5,6]. Venom disintegrins have received a great deal of attention owing to their ability to bind to integrins in cell membranes, inducing a variety of effects which largely depend on the identity of the target integrin. In turn, integrins comprise a superfamily of receptors that play critical roles in the processes of cell–cell adhesion and cell-matrix adhesion, platelet aggregation, inflammatory reactions, cell migration, angiogenesis, cell signaling, and others [7,8]. These proteins are heterodimers of transmembrane α and β subunits [9]. Various extracellular matrix and plasma proteins serve as ligands for integrins, such as fibronectin,

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vitronectin, fibrinogen, laminin, some types of collagen, and von Willebrand factor. Several of these molecules interact with integrins through an internal sequence, which often comprises the tripeptide RGD [7] or the triple-helical GFOGER sequence in the major collagens [9]. In the case of endothelial cells, integrins are essential for their adhesion to the basement membrane, thus contributing to the integrity and stability of blood vessels [8]. Integrin $\alpha_5\beta_1$ functions as a fibronectin receptor, whereas integrin $\alpha_3\beta_1$ binds to laminin, collagen and fibronectin; integrin $\alpha_2\beta_1$ is a collagen and laminin receptor, and integrin $\alpha_v\beta_3$ is a vitronectin receptor [10,11]. Integrin $\alpha_{IIb}\beta_3$ occurs in the plasma membrane of platelets, where it contributes to platelet aggregation after the binding to fibrinogen or von Willebrand factor [12,13].

Snake venom disintegrins have been classified into four different groups according to their polypeptide length and the number of disulfide bonds [14,6]. The first group comprises the long disintegrins with 84 amino acids and 7 disulfide bonds [15]. The second group is formed by medium-size disintegrins containing about 70 amino acids and 6 disulfide bonds; the majority of disintegrins characterized belong to this group. The third group is composed of homodimeric and heterodimeric molecules. Dimeric disintegrins contain subunits of about 67 residues with 10 cysteines involved in the formation of 4 intra-chain disulfide bonds and 2 interchain cystine linkages [16–18]. The fourth group is constituted by short disintegrins composed of 41–51 residues and 4 disulfide bonds [19,20].

The binding of disintegrins to integrins is primarily mediated by a sequence of three amino acids located in a loop near the C-terminus [21–23]; such interaction precludes the binding of integrins to their physiological ligands in the extracellular matrix. The majority of disintegrins contain the sequence RGD which represents the ancestral integrin-recognition motif [6,22,24,25]. The disulfide bonds around the RGD sequence determine the formation of a loop [20,26–29]. Therefore both the amino acids adjacent to the RGD tripeptide and the location of disulfide bonds determine the conformation and, consequently, the affinity of the disintegrin for receptors [15,25,30,31]. RGD disintegrins bind to the αIIbβ3 integrin on the platelet membrane, thus inhibiting platelet aggregation [14]; moreover, RGD blocks integrins $\alpha_8\beta_1$, $\alpha_5\beta_1$,$\alpha_v\beta_1$ and $\alpha_v\beta_3$. Some disintegrins contain variations in the RGD sequence, generated by at least three mutations, such as KGD, that inhibits $\alpha_{IIb}\beta_3$ integrin, MGD and VGD, which bind to integrin $\alpha_5\beta_1$, and WGD, that binds to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. In addition, MLD targets $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_9\beta_1$ integrins and is characteristic of heterodimeric disintegrins [23,31–35]. The conserved aspartate residue of these tripeptides might be responsible for the binding to the β subunit of integrins, whereas the other two residues determine the binding to the α subunit [36].

Another group of disintegrins, characterized by the sequences KTS and RTS, is comprised by short, monomeric molecules containing about 40 amino acids in its polypeptide chain; they bind $\alpha_1\beta_1$ integrins [35,37–39]. The threonine present in the tripeptide KTS of the disintegrin obtustatin is key for the binding to $\alpha_1\beta_1$ integrin [40]. It has been suggested that the RTS/KTS short disintegrins may have been recruited into the venom gland of Eurasian vipers independently of the canonical neofunctionalization pathway characteristic of the RGD disintegrins [41].

In addition to the well-characterized effect of inhibition of platelet aggregation, through the binding to $\alpha_{IIb}\beta_3$ integrin, other activities of potential pharmacological applications have been described for RGD disintegrins. For example, interaction with integrin $\alpha_v\beta_3$ affects cell migration, with impact in angiogenesis, tumor metastasis, and atherosclerosis [42,43]. Various disintegrins have been shown to reduce experimental metastasis in melanomas [44,45], and others inhibit endothelial cell adhesion to the extracellular matrix [25]. Thus, the broad pharmacological scope of disintegrins underscores the need for a continuous exploration in search of novel disintegrins and the identification of their targets and pharmacological activities. The proteomes of venoms from Central American viperid species have been studied in the last years [46–53]. This intensive characterization has highlighted the presence of disintegrins in the venoms of several of these species, thus raising the possibility of isolating and characterizing novel members of this family of proteins from these venoms. In the present work we describe the isolation and structural and functional characterization of three novel disintegrins from the venoms of the Mesoamerican species *Atropoides mexicanus*, *Cerrophidion sasai* and *Crotalus simus*. In addition, the characterization of a disintegrin previously isolated from the venom of *Bothrops asper* [54] is now described.

2. Materials and methods

2.1. Venoms

The venoms of *A. mexicanus*, *B. asper*, *C. simus* and *C. sasai* were obtained and pooled from adult specimens collected at various locations in Costa Rica and kept at the serpentarium of Instituto Clodomiro Picado, Universidad de Costa Rica. Immediately after extraction, the venoms were centrifuged to remove insoluble debris, lyophilized and stored at −20 °C.

2.2. Purification of disintegrins

Batches of 20 mg of freeze-dried venoms from *A. mexicanus*, *B. asper*, *C. simus* and *C. sasai* were dissolved in 250 μL of solution A (5% acetonitrile, 94.9% water and 0.1% trifluoroacetic acid). Insoluble material was discarded after centrifugation at 3000 × *g* for 3 min, and the clear supernatants were fractionated by reverse-phase HPLC on a Vydac C-18 (250 × 10 mm; 5 μm particle size) using an Agilent Model 1200 chromatograph, eluting at a flow rate of 2 mL/min with a gradient that utilized 0.1% TFA and 5% acetonitrile in water (solution A) to 0.1% TFA in acetonitrile (solution B), as follows: 0–15% B over 30 min, 15–20% B to 42 min, 20–80 % B to 43 min, maintained at 80% B to 59 min, and 0% B to 60 min. Separations were monitored at 215 nm, and fractions were collected manually and dried using a Speed-Vac system (Thermo Scientific, Minneapolis, USA). HPLC-separated fractions were dissolved in water and their final protein concentration was determined with the "DC Protein Assay" (BioRad, California, USA) using bovine serum albumin as standard.

2.3. Homogeneity and molecular mass determination

Homogeneity of the final preparations was evaluated by electrophoresis on a discontinuous triphasic polyacrylamide gel system [55], in the presence of sodium dodecylsulphate and tricine (SDS-PAGE), with a 4% spacer gel, a 10% first separating gel, and a 16.5% second separating gel. A mixture of low molecular weight markers was included. Separations were performed with a constant voltage of 120 V for 1–2 h, and the proteins were visualized by staining with Coomassie Blue R-250. The molecular mass of each isolated disintegrin was determined by MALDI-TOF on a model 4800-Plus Proteomics Analyzer (Applied Biosystems). The proteins were mixed with an equal volume of a saturated solution of α-cyano-hydroxycinnamic acid in 50% acetonitrile and applied (1 μL) onto an OptiTof-384 plate (ABSciex). After drying, the samples were analyzed in linear positive mode using 500 shots/spectrum and laser intensity of 4200, in the *m*/*z* range 2000 to 18,000. Calibration