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Molecular characterization of metalloproteases from *Bothrops alternatus* snake venom

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ABSTRACT

We have previously demonstrated that alternagin-C (ALT-C), a disintegrin-like, Cys-rich protein isolated from *Bothrops alternatus* snake venom, induces human vascular endothelial cell (HUVEC) proliferation and angiogenesis in in vitro and in vivo assays. Therefore this protein could be interesting as a new approach for tissue regeneration studies. However, its primary sequence was not completely determined since the protein isolated from crude venom is usually a mixture of isoforms. Here we describe the transcriptome analysis of *B. alternatus* from the venom glands of a single male specimen. About 800 good-quality contigs were screened for snake venom metalloproteases/disintegrins, resulting in the following expression profile for these enzymes: 4% for P-I, 7% for P-II and 89% for P-III SVMPs. The PII-SVMP sequence code for RGD-disintegrins and all the expressed PIII-sequences have the ECD adhesive motif. A cDNA sequence coding for an ALT-C homolog was completely sequenced and characterized. Comparative sequence and structural analyses suggested new features that distinguish SVMP classes such as two prolyl endopetidase cleavage sites. All these data add new information on the expression pattern of metalloproteases of *B. alternatus* venom and may have practical applications for the production of recombinant disintegrins for cell adhesion studies.

1. Introduction

Snake venom metalloproteases (SVMPs) comprise a family of highly complex but conserved proteins found in most Viperidae venom. The SVMPs are classified as members of the adamalysin family of the metzincin clan of the metalloendopetidase super family (Takeda et al., 2012). The SVMPs are Zn^{2+}-dependent enzymes which play central roles in venom toxicity by degrading the extracellular matrix (ECM) components such as collagen, laminin and fibronectin, resulting in hemorrhage, edema and necrosis in affected victims (Bjarnason and Fox, 1994). The SVMPs can also activate endothelial cells, and inhibit both blood coagulation and platelet aggregation (Moura-da-Silva et al., 2007).

The SVMPs are synthesized as inactive precursor forms due to the presence of a highly conserved motif PKMCGVT known as cysteine switch in the proenzyme domain (Grams et al., 1993). The SVMPs are multimodular proteins that are divided into three classes (PI to PIII) accordingly to their molecular mass and domain organization (Fox and Serrano, 2008). Members of the PI class are represented by the catalytic domain only, while the PII and PIII classes have additional C-terminal adhesive domains linked to the proteolytic domain. The catalytic domain is characterized by the presence of a conserved zinc-binding consensus sequence HEXXHXXGXXHD (Hooper, 1994). The members of the PII class are characterized by the presence of a disintegrin domain, where an adhesive tripeptide motif such as RGD, VGD, or KGD, among others, is found (Calvete, 2013). The SVMPs of PIII class have instead a disintegrin-like domain with E/DCD adhesive motif, followed by a Cys-rich domain. After enzyme activation during venom secretion, some SVMPs undergo proteolysis of such domains, releasing the free disintegrin domain or the disintegrin-like domain linked to the Cys-rich domain (DC domain). However, there are also SVMPs that do not release the DC domain and may be purified from the venom as full P-III SVMPs (Moura-da-Silva et al., 2003). Additional complexity is also given by post-translational modifications such as domain dimerization resulting in at least eleven subclasses of SVMPs (Fox and Serrano, 2008). Such diversity has been suggested to be derived by accelerated evolution (Moura-da-Silva et al., 1996), post-

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translational modifications (Fox and Serrano, 2008), and more recently, by genetic recombination between the SVMP classes (Moura-da-Silva et al., 2011). However, the first description of the genomic organization of a PIII SVMP gene suggesting a complex exon–intron organization gave additional evidence of the complexity of the SVMPs (Sanz et al., 2012).

The processed disintegrin domains bind to integrin receptors in platelet or cell surfaces with high affinity and specificity therefore inhibiting platelet aggregation or cell adhesion to the ECM (Gould et al., 1990). Inhibition of cell adhesion is particularly relevant in metastasis therapy since tumor cells need to bind to many substrates such as the endothelium or ECM components in order to achieve a secondary site. In this way, the integrins were considered as important targets for metastasis prevention since integrin inhibition blocks cell adhesion to the ECM (Felding-Habermann et al., 2001). A few integrin blockers have entered in clinical trials for cancer therapy with encouraging results such as cilengitide, an inhibitor of the vitronectin receptors, the αvβ3 and αvβ5 integrins (Desgrosellier and Cheresh, 2010).

Alternagin-C (ALT-C), a disintegrin-like-Cys-rich protein isolated from the venom of the Brazilian snake *Bothrops alternatus* (Souza et al., 2000) binds specifically to the integrin α2β1, one of the major collagen receptors in several cell types. ALT-C induces in vitro migration of human neutrophils by triggering classical integrin-mediated intracellular signaling including FAK and IP3K phosphorylation, Erk translocation to the nucleus and actin polymerization, crucial steps for the cell migration (Mariano-Oliveira et al., 2003). In addition, ALT-C is a strong inducer of HUVEC proliferation in vitro, by up-regulating the expression of VEGF (vascular endothelial growth factor) and of its receptor VEGFR 2 (vascular endothelial growth factor receptor 2) (Cominetti et al., 2004). DisBa-01 is a recombinant RGD-disintegrin from *B. alternatus* venom that inhibits αvβ3 integrin binding (Ramos et al., 2008) as well as cell migration (Selistre-de-Araujo et al., 2010). DisBa-01 has potent antithrombotic activity (Kauskot et al., 2008) and it was shown to inhibit experimental metastasis in nude mice (Ramos et al., 2008).

To contribute for a better understanding of SVMP complexity, we constructed a cDNA library from the venom glands of a single specimen of *B. alternatus*, characterized its tissue expression pattern and identified the sequence of several metalloproteases. This data may have practical applications in the design of new integrin-binding drugs for modulation of integrin activity.

Table 1
Comparative analysis of the two transcriptomes from *B. alternatus*.

	Cardoso et al. (2010)	This work
Sequenced ESTs (tn*)	5350	1920
Singlets (%)		33
Contigs (%)		67
No hits ESTs (%**)	70	21
Cell processs ESts (%)	7	18
Toxic ESTs (%)	23	61
SVMPs, total (%***)	81	59
PI SVMP (%****)	0	4
PII SVMP (%****)	nd	7
PIII SVMP (%****)	nd	89
BBPs (%***)	8	12
C-type lectins (%***)	1.5	16
PLA2s (%***)	5.6	1.5
Serine proteases (%***)	2	5
Other transcripts (%***)	2	6.5

*tn = total number; **% from the total sequenced ESTs; *** % from toxic ESTs; **** from SVMP ESTs; nd = not determined.

2. Materials and methods

2.1. Venom gland extraction

One male specimen of *B. alternatus* (family *Viperidae*, subfamily *Crotalinae*) was kindly provided by Prof. Dr. Augusto Shinya Abe (Department of Zoology, UNESP — Rio Claro, Brazil). Three days before the removal of the glands the venom was collected for increasing mRNA synthesis. The glands were extracted and kept on liquid nitrogen in TRIzol reagent (Invitrogen®) until use.

2.2. RNA extraction and mRNA isolation

Total RNA from the venom gland was extracted by the method previously described (Chomczynski and Sacchi, 1987) slightly modified;

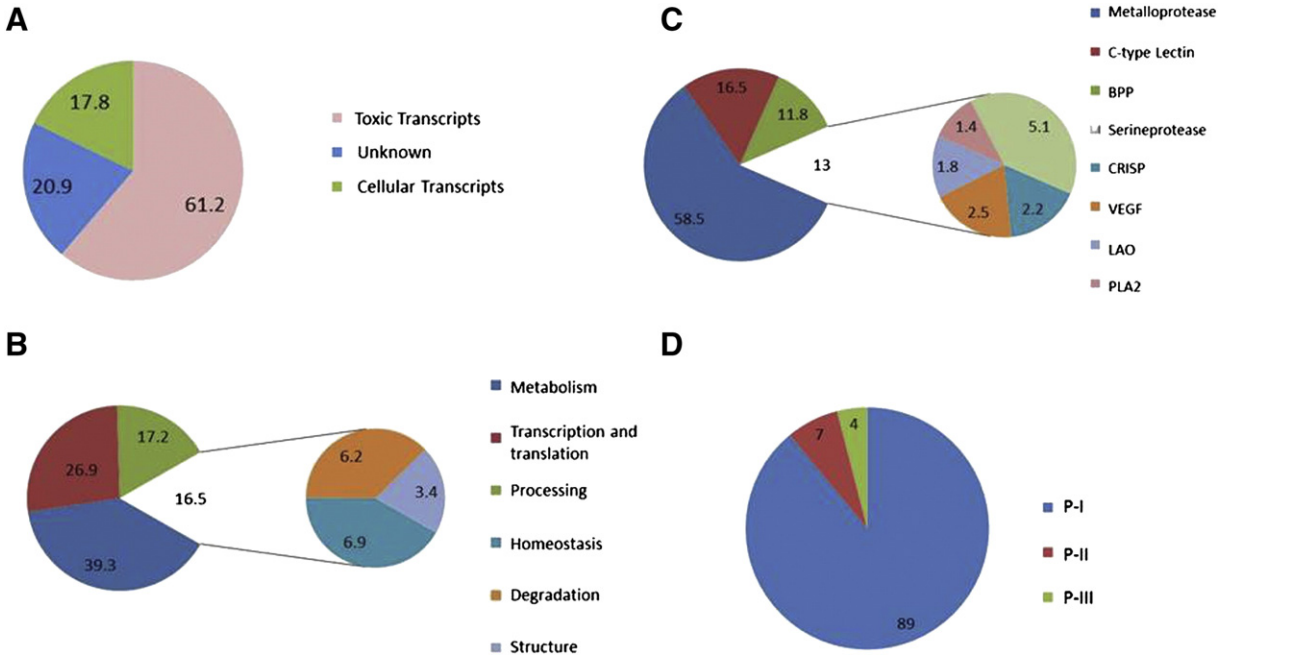

Fig. 1. Transcripts in *Bothrops alternatus* venom gland. A) General distribution; B) transcripts related to cellular physiology; C) transcripts related to toxins; and D) percentage of each SVMP class among the metalloprotease transcripts.