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Mapping von Willebrand factor A domain binding sites on a snake venom metalloproteinase cysteine-rich domain

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Abstract

The PIII class of the snake venom metalloproteinases (SVMPS) are acknowledged to be one of the major hemorrhage producing toxins in crotalid venoms. This class of SVMPS are structurally distinguished by the presence of disintegrin-like and cysteine-rich domains carboxy to the metalloproteinase domain and thus share structural homology with many of the ADAMs proteins. It has been suggested that the presence of the carboxy domain are the key structural determinants for potent hemorrhagic activity in that they may serve to target the proteinases to specific key extracellular matrix and cell surface substrates for proteolysis leading to hemorrhage production at the capillaries. Following from previous studies in our laboratory in this investigation we scanned the cysteine-rich domain of the PIII hemorrhagic SVMP jararhagin using synthetic peptides in an attempt to identify regions which could bind to von Willebrand factor (vWF), a known binding partner for jararhagin. From these studies we identified two such peptide, Jar6 and Jar7 that could support binding to vWF as well as block the recombinant cysteine-rich domain of jararhagin binding to vWF. Using the coordinates for the recently solved crystal structure of the PIII SVMP VAP1, we modeled the structure of jararhagin and attempted to dock the modeled cysteine-rich structure of that protein to the A1 domain of vWF. These studies indicated that effective protein–protein interaction between the two ligands was possible and supported the data indicating that the Jar6 peptide was involved, whereas the Jar7 peptide was observed to be sterically blocked from interaction. In summary, our studies have identified a region on the cysteine-rich domain of a PIII SVMP that interacts with vWF and based on molecular modeling could be involving in the interaction of the cysteine-rich domain of the SVMP with the A1 domain of vWF thus serving to target the toxin to the protein for subsequent proteolytic degradation.

Keywords: Snake venom metalloproteinases; von Willebrand factor; Cysteine-rich domain; SVMP; vWF A domains

The local and systemic hemorrhage resulting from envenomation by crotalid snakes is in large part due to the hemorrhagic metalloproteinases present in these venoms [1]. In general it is considered that hemorrhage occurs following proteolytic degradation of extracellular matrix proteins and endothelial cell surface proteins involved in the maintenance of capillary structural and functional integrity [1]. The snake venom metalloproteinases (SVMPs)[1] responsible for this degradation comprise a subgroup of the reprolysin subfamily of the M12 class of metalloproteinases [2]. The classification of these enzymes is based on size and different domain structures: PI SVMPs have only a metalloproteinase domain; PII have metalloproteinase and disintegrin domain; PIII SVMPs are synthesized with metalloproteinase, disintegrin-like and cysteine-rich domain and PIV have the PIII domain structure plus lectin-like domains connected by disulfide bonds [1]. The PIII class, the most potent SVMP class, is related to the ADAM (A Disintegrin And Metalloproteinase) protein family, which contains besides the metalloproteinase, disintegrin-like and cysteine-rich domains an epidermal growth factor-like region, a transmembrane region and a cytoplasmic tail [3].

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[1] *Abbreviations used:* SVMP, snake venom metalloproteinase; ADAM, a disintegrin and metalloproteinase; RP-HPLC, reversed phase-high performance liquid chromatography.

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Closely related to the ADAMs are the multidomain, extracellular ADAMTS proteins. ADAMTS proteins are distinguished from the ADAMs proper primarily by the fact that they lack the transmembrane and cytoplasmic domains of the ADAMs and have one or more thrombospondin motif domains [4]. One member of the ADAMTS family, ADAMTS-13, has been observed to play a critical role in human hemostasis by binding to and proteolytically degrading multimeric vWF to small complexes which are less able to spontaneously give rise to thrombi [5,6]. Genetic lesions in ADAMTS-13 which abolish or decrease its ability to degrade large multimeric vWF leads to the disease thrombotic thrombocytopenic purpura distinguished by microvascular vWF and platelet rich thrombi leading to anaemia, renal failure and neurological dysfunction [7,8]. Several studies have shown that ADAMTS-13 interaction with vWF is via its disintegrin-like/cysteine-rich domain and this interaction is necessary for effective degradation of vWF [9]. Similar studies have shown that the PIII SVMPs are also capable of binding to and proteolytically cleaving vWF [10,11].

In light of the fact that the PIII class display the greatest hemorrhagic potency of the SVMPs there have been several studies investigating the role of the disintegrin-like/cysteine-rich domains, the distinguishing features of this class. Usamai and colleagues demonstrated that jararhagin-C, the disintegrin-like/cysteine-rich domains proteolytic product from the PIII hemorrhagic SVMP jararhagin, isolated from the venom of *Bothrops jararaca* inhibited collagen and ADP-stimulated platelet aggregation [12]. This activity for the disintegrin-like/cysteine-rich domains of PIII SVMPs was corroborated by Jia and colleagues who showed that a recombinant disintegrin-like/cysteine-rich domain containing protein based on atrolysin A, a PIII hemorrhagic SVMP from *Crotalus atrox*, was capable of inhibiting collagen-stimulated platelet aggregation [13]. The capability of the cysteine-rich domain alone to recapitulate the activity of the disintegrin-like/cysteine-rich domains for inhibiting platelet aggregation was demonstrated by Jia and colleagues using a recombinant cysteine-rich domain from atrolysin A [14]. Recently, these studies were extended to shown that the cysteine-rich domain of PIII SVMPs was responsible for the interaction to, and presumably the degradation of von Willebrand factor (vWF) [15]. These data indicated a functional role of the cysteine-rich domain of the PIII SVMPs in targeting them to proteins involved in platelet aggregation such as the $\alpha 2\beta 1$ integrin and vWF. Recent studies from our laboratory have shown that the cysteine-rich domain of the jararhagin is capable of targeting other proteins which contain vWF A domains, such as collagen XII and XIV and matrilins, for proteolytic degradation (under review for publication). Finally, the crystal structure of a PIII SVMP, vascular apoptosis protein 1, from *Crotalus atrox*, was recently reported [16]. From the crystal structure of VAP1 it was clearly observed that the ECD sequence motif, which has been suggested to be involved in integrin binding by this domain, was sterically unavailable for interaction. Thus the site(s) of interaction with integrins likely reside elsewhere with the protruding cysteine-rich domain at the carboxy region of the structure an obvious candidate [16].

In this study, we describe the use of synthetic peptides spanning the cysteine-rich domain of the hemorrhagic PIII SVMP jararhagin to localize the site of interaction on this domain with vWF. Using this approach we identified two peptides capable of binding to vWF and blocking the binding of recombinant jararhagin cysteine-rich domain to vWF. Mapping of these peptides on the structure of the cysteine-rich domain of VAP1 suggest that only the region in the domain represented by the peptide Jar6 is sterically available for protein–protein interaction. In addition, we show via molecular modeling that the cysteine-rich domain of jararhagin could potentially interact with the A1 domain of vWF supported in part by the interaction deliminated by the Jar6 peptide.

Materials and methods

Peptide synthesis

Peptides were designed based on the sequence of the cysteine-rich domain of jararhagin, a PIII SVMP from *Bothrops jararaca* venom. All peptides were synthesized on a Symphony multiple peptide synthesizer (Rainin) using Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) chemistry according to the manufacture's recommendation. Peptides were purified to homogeneity by RP-HPLC (reversed phase-high performance liquid chromatography) and desalted by gel filtration chromatography. Following purification the peptides, all of which had cysteinyl residues near the N- and C-terminal were cyclized by air oxidation as previously described [13].The peptides were then analyzed by MALDI-TOF mass spectrometry to confirm the correct mass and cyclization of the peptide. Lyophilized peptides were dissolved in HBS-EP buffer [10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% (v/v) surfactant P20; BIAcore™] to final concentration of 1 μg/μl immediately prior to use. One cyclized peptide, Jar6, was also reduced and carboxyamidomethylated [15].

Expression of recombinant atrolysin a cysteine-rich domain

The expression vector for the cysteine-rich domain of atrolysin A (A/C) was constructed as described elsewhere [15]. Briefly, cDNA fragment of atrolysin A (301–413) was amplified by PCR from the plasmid pMbacA/C and subcloned into the pET102/D-TOPO bacterial expression vector (Invitrogen). BL21 Star (DE3) *Escherichia coli* cells (Invitrogen) were transformed by heat shock with the plasmid pET102/D-A/C, according to the manufacturer's instructions and grown overnight at 37 °C. Bacterial cells were inoculated in LB and expression of recombinant proteins was induced by 1 mM IPTG (isopropyl *b*-D-thiogalactoside) for 3 h at 37 °C. Cell pellet was lysed by French press in lysis buffer [50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 0.1 mg/ml lysozyme, 10 mM imidazole and 1 mM PMSF], and resulting suspension centrifuged at 10000*g* for 30 min at 4 °C. Soluble fraction was applied onto a Talon metal affinity column (Clontech). The column was washed with binding buffer and bound proteins were eluted with binding buffer containing 0.15 M imidazole. Protein homogeneity was determined by SDS–PAGE and MALDI-TOF mass spectrometry.

Surface plasmon resonance assays

Protein–peptide and protein–protein interactions were studied by surface plasmon resonance using the BIAcore™ 3000 system, at 25 °C. vWF