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Characterization of Neuwiedin, a new disintegrin from *Bothrops neuwiedi* venom gland with distinct cysteine pattern

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ABSTRACT

Disintegrins are cysteine-rich toxins containing the RGD motif exposed in a loop that binds integrins such as $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$. The flexibility of the RGD loop, controlled by the profile of the cysteine pairs and the residues flanking the RGD sequence, are key structural features for the functional activity of these molecules. Recently, our group reported a transcript in the venom gland of *Bothrops neuwiedi* corresponding to a new P-II SVMP precursor, BnMPIIx, in which the RGD-binding loop includes many substituted residues and unique cysteine residues at the C-terminal. In this paper, we obtained the recombinant disintegrin domain of BnMPIIx, Neuwiedin, which inhibited ADP-induced platelet aggregation, endothelial cell adhesion to fibrinogen and tube formation in Matrigel with no particular selectivity to $\alpha_{IIb}\beta_3$ or endothelial cell integrins. This value was also comparable to the inhibition observed with other recombinant disintegrins with conserved cysteine positions and residues in RGD loop. In this regard, Neuwiedin is an important component to understand the functional relevance of the diversity generated by accelerated evolution of venom toxins as well as to find out eventual new disintegrin-dependent targets that may be approached with disintegrins.

1. Introduction

A common feature in snake venoms is the presence of homologous proteins, with similar structural scaffold, but different biological activities, as frequently observed in snake venom disintegrins (Calvete et al., 2005b; Gomis-Rüth, 2003; Lu et al., 2005; Tsai et al., 2004). Disintegrins are a group of low molecular mass proteins present in the venoms of viper snakes by proteolytic processing of PII-class metalloproteinase precursors (Calvete, 2010; Fox and Serrano, 2008). Classical disintegrins contain the RGD motif exposed in a loop that binds to RGD-dependent integrins such as $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ expressed on surface of different cell types, particularly platelets, vascular endothelial cells and some tumor cells, leading to inhibition of platelet aggregation or cell adhesion, migration and angiogenesis (Calvete, 2013; Huang et al., 2001). Due to their affinity to integrins, disintegrins have found numerous applications in studies on a variety of biological processes in which integrins play pivotal roles (Marcinkiewicz, 2007, 2013; Niewiarowski et al., 2002).

Disintegrins are classified according to the pattern of disulfide bonds, which is highly conserved within each subclass due to evolutionary correlations (Calvete et al., 2005a; Carbajo et al., 2015). This group of toxins evolved from ancestral physiological proteins (ADAMs) recruited to the venom and their evolutionary pathway passes through a process involving the loss of cysteine pairs and reduction of the N-terminal region (Juárez et al., 2008). Thus, according to their structure, disintegrins may be classified according to their length and number of disulfide bonds into long, medium sized, dimeric, and short toxins (Calvete et al., 2003). Monomeric disintegrins are also grouped according to the number of cysteines: short disintegrins contain 8 cysteines, while medium and long disintegrins contain 12 and 14 cysteines, respectively. Dimeric disintegrins contain 10 cysteines in each subunit (Marcinkiewicz, 2013). Other relevant structural motifs are present in disintegrin binding loops and they modulate the selectivity of disintegrins towards cellular responses including leukocytes, endothelial cells and tumor cells: MLD motif has been found exclusively in heterodimeric disintegrins that interact with $\alpha_4\beta_1$

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present in T-lymphocytes and $\alpha_9\beta_1$ present in neutrophils and endothelial cells (Bazan-Socha et al., 2004; Coelho et al., 2004; Marcinkiewicz et al., 1999, 2000; Saldanha-Gama et al., 2010; Walsh et al., 2012). The WGD motif on CC8 heterodimeric disintegrin, isolated form *Cerastes cerastes* venom, the strongest blocker of fibrinogen binding, however with decreased selectivity (Calvete et al., 2002). The KTS motif are referred to block eosinophils adhesion and migration by $\alpha_1\beta_1$ integrin (Bazan-Socha et al., 2012) and act in endothelial cells by inducing apoptosis and blocking adhesion to collagen (Brown et al., 2009; Olfa et al., 2005). Amino acids residues adjacent to the RGD motif within their integrin-binding loops also modulate the interaction with integrins. Monomeric disintegrins show different levels of affinity and selectivity toward the $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins depending on the other residues located in the loop (Lu et al., 1996; Marcinkiewicz et al., 1997; Scarborough et al., 1993; Wierzbicka-Patynowski et al., 1999). The potency of disintegrins may be also dependent on the residue C-terminally adjacent to the active motif (Brown et al., 2009). However, Pfaff et al. (1994) demonstrated that disintegrins that have identical amino acid residues after RGD sequence do not necessarily show the same integrins selectivity. The integrin-inhibitory activity of disintegrins critically depends on the appropriate pairing of cysteine residues, which determines the conformation of the inhibitory loop (Niewiarowski et al., 1994). Rahman et al. (2000) demonstrated a negative allosteric relationship between specific RGD motifs and distinct sites of RGD ligand recognition on the $\alpha_{IIb}\beta_3$ complex. Therefore, the flexibility in the RGD loop may be important to access a number of different conformations required for binding (Lazarus and McDowell, 1993).

Recently, our group showed a great diversity of cDNAs encoding SVMPs in a single venom gland of a *Bothrops neuwiedi* specimen. The most complex group was the class P-II SVMPs represented by three independent subclasses with different degrees of homology with other classes of SVMPs and distinct positions for cysteine residues. This includes a new P-II SVMP precursor, BnMPIIx, composed of a combination of a typical P-III catalytic domain and an RGD-disintegrin domain with many substituted residues in the RGD-binding loop and unique cysteines present at the C-terminal (Moura-da-Silva et al., 2011). Continuing the study with SVMPs from *B. neuwiedi*, recently, Bernardoni et al. (2014) confirmed that SVMPs isolated from this venom were selective to different targets, acting with distinct potency on mammalian or bird blood, emphasizing, thus, the importance of functional diversity of these molecules and the disintegrins released from them. The structural uniqueness of the cysteine pattern present in the disintegrin generated from BnMPIIx precursor and the peculiarity of the amino acid substitutions, motivated us to clone and express this new disintegrin from *B. neuwiedi* to evaluate its biological activity. For this, the disintegrin-coding cDNA was cloned in fusion with Small Ubiquitin-like Modifier (SUMO) in order to obtain this toxin in the soluble form in the bacteria cytoplasm. After SUMO removal from disintegrin, its inhibition of platelet aggregation and endothelial cell adhesion were evaluated, indicating important issues to be included for understanding the molecular evolution of snake venom disintegrins.

2. Material and methods

2.1. Cloning and expression of Neuwiedin in Escherichia coli

The cDNA template used in this work was synthesized from a messenger RNA isolated from the venom gland of the snake *B. neuwiedi* (Moura-da-Silva et al., 2011) and cloned into pGEM-T vector. This sequence was named BnMPIIx2 and deposited in GenBank under accession number HM443640. The cDNA sequence coding for the disintegrin domain of BnMPIIx2 (Neuwiedin) was amplified by PCR using primers comprising the 5′ and 3′ ends of the disintegrin domain in which endonucleases sites *Bam*HI and *Hind*III (underline) were added, respectively. The forward and reverse primers were as follow: GTAAGGATCCGGAGGTGGGAGAAGAA; GCAGGAAGCTTTTAGCCACAGAAGTGAAA, respectively. The PCR product was digested and purified by Wizard® PCR Purification Kit (Promega) and cloned in frame with *Small Ubiquitin-like Modifier* (SUMO) sequence into pMST3 vector, a modified pET28b vector with SUMO sequence cloned 3′ to the His6 tag (Yunus and Lima, 2009). This construction was denominated pMST3-Neuwiedin. The pMST3-Neuwiedin was used to transform chemically competent *Escherichia coli* C43 (DE3) cells which were overnight cultured into LB broth containing 100 μg/mL of ampicillin. The overnight culture was then used to inoculate 250 mL of fresh LB (100 μg/mL ampicillin) medium at 1:100 dilution. At an optical density of 0.6 (600 nm), isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was maintained for 4 h before the cells were harvested by centrifugation at 5000 g for 10 min at 4 °C. Cells were resuspended in binding buffer (50 mM sodium phosphate; 300 mM NaCl; 10 mM imidazole; pH 7.0) and intermittently sonicated on ice for 60 s with intervals of 2 min for cooling, with total sonication time of 6 min followed by centrifugation at 7000 g, 10 min, 4 °C. The recombinant protein was purified from the supernatant by immobilized metal affinity chromatography (IMAC) using Ni Sepharose® High Performance (GE Healthcare) following the manufacturer's recommendations. After purification, the recombinant protein was dialyzed against Tris buffer (50 mM Tris; 100 mM NaCl; 1 mM DTT; 1% glycerol; pH 8.0) and analyzed in a 12% SDS-PAGE under reducing conditions.

2.2. Ulp1 expression and SUMO cleavage

The His-tagged N-terminal fragment (amino acid residues 403–621) from SUMO protease 1, $Ulp1_{403\text{-}621}$, from *Saccharomyces cerevisiae*, cloned into pET28a (Mossessova and Lima, 2000) was expressed in BL21 Star™ (DE3)pLysS (Invitrogen) following the same protocol described for Neuwiedin expression. The recombinant protein was purified using Ni Sepharose® High Performance (GE Healthcare) following the manufacturer recommendations. After purification the recombinant protein was dialyzed against Tris buffer (50 mM Tris; 100 mM NaCl; 1 mM DTT; 1% glycerol; 1% Triton X-100; pH 8.0). Neuwiedin fused to SUMO was then incubated with purified Ulp1 at the ratio of 10:1 in Ulp1 buffer (500 mM Tris–HCl; 2% triton X-100, 10 mM DTT; pH 8.0) for 6 h at 30 °C. After cleavage, the His-tag containing fragments of ULP1 and SUMO were removed by incubation with Ni Sepharose® High Performance (GE Healthcare) under rotation for 30 min. The resin was then removed by centrifugation for 5 min at 800 g and the supernatant, containing Neuwiedin free from SUMO, was collected. Neuwiedin concentration was evaluated using Micro BCA™ Protein Assay Kit (Pierce).

The molecular mass of the recombinant protein was checked by mass spectrometry. Neuwiedin (50 μg) purified by IMAC was incubated with 10 mM DTT for 45 min at 60 °C. The sample was cooled at RT and 20 mM iodoacetamide (IAM) was added (Thermo Scientific) followed by incubation for 30 min in dark. After this, 5 mM DTT were added to quench the alkylation. The sample was desalted using Zeba Micro Spin Desalting Columns (thermo Scientific) according to the manufacturer's instructions. The mass spectrometry analysis (MALDI-TOF MS) was performed on AutoflexSpeed (Bruker) equipment on a linear mode calibrated with insulin, cytochrome c, myoglobin, ubiquitin I and aldolase. The sample was mixed in a 1:1 ratio with a matrix consisting of sinapinic acid (10 mg/mL) in 50% acetonitrile and 0.1% TFA, and applied on the MALDI plate using the dried-droplet method.