A prothrombin activator from *Bothrops erythromelas* (jararaca-da-seca) snake venom: characterization and molecular cloning

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A novel prothrombin activator enzyme, which we have named 'berythractivase', was isolated from *Bothrops erythromelas* (jararaca-da-seca) snake venom. Berythractivase was purified by a single cation-exchange-chromatography step on a Resource S (Amersham Biosciences) column. The overall purification (31-fold) indicates that berythractivase comprises about 5% of the crude venom. It is a single-chain protein with a molecular mass of 78 kDa. SDS/PAGE of prothrombin after activation by berythractivase showed fragment patterns similar to those generated by group A prothrombin activators, which convert prothrombin into meizothrombin, independent of the prothrombinase complex. Chelating agents, such as EDTA and *o*-phenanthroline, rapidly inhibited the enzymic activity of berythractivase, like a typical metalloproteinase. Human fibrinogen Aα-chain was slowly digested only after longer incubation with berythractivase, and no effect on the β- or γ-chains was observed. Berythractivase was also capable of triggering endothelial proinflammatory and procoagulant cell responses. von Willebrand factor was released, and the surface expression of both intracellular adhesion molecule-1 and E-selectin was up-regulated by berythractivase in cultured human umbilical-vein endothelial cells. The complete berythractivase cDNA was cloned from a *B. erythromelas* venom-gland cDNA library. The cDNA sequence possesses 2330 bp and encodes a preproprotein with significant sequence similarity to many other mature metalloproteinases reported from snake venoms. Berythractivase contains metalloproteinase, desintegrin-like and cysteine-rich domains. However, berythractivase did not elicit any haemorrhagic response. These results show that, although the primary structure of berythractivase is related to that of snake-venom haemorrhagic metalloproteinases and functionally similar to group A prothrombin activators, it is a prothrombin activator devoid of haemorrhagic activity. This is a feature not observed for most of the snake venom metalloproteinases, including the group A prothrombin activators.

Key words: berythractivase, coagulation, haemostasis, metalloproteinases, thrombosis.

INTRODUCTION

Snake venoms contain a complex mixture of potentially toxic and non-toxic components that have wide-ranging pathological effects on vital functions, including blood coagulation, the cardiovascular system, renal function, fibrinolysis and complement systems [1,2]. Many snake venoms have been shown to contain one or more procoagulant activities. Activators of prothrombin have been isolated from the venoms of a wide variety of snake species, particularly those belonging to family Viperidae. There are, however, large differences between the mechanisms by which these venom components activate prothrombin [3–5]. According to its distinct structural and functional properties, snake-venom prothrombin activators have been classified into four groups [3,6]. Kini et al. [7] proposed a new classification as groups A, B, C and D. Group A includes metalloproteases that efficiently activate prothrombin without the requirement of any cofactors, such as Ca^{2+}, phospholipids or Factor Va, e.g. activators from *Echis carinatus* (Kenya carpet viper or saw-scaled viper) [8], *Bothrops atrox* (fer-de-lance) [9] and *Bothrops neuwiedi* (Neuwied's lancehead or Maximillian's viper) [10]. Group A proteins cleave the Arg^{320}–Ile^{321} bond in prothrombin and produce meizothrombin, which is converted into α-thrombin by autolysis. Group B contains Ca^{2+}-dependent metalloproteases such as carinactivase-1 from *E. carinatus leukogaster* (white-bellied saw-scaled viper) [11] and multiactivase from *E. multisquamatus* (Central Asian sand viper) [5]. In contrast with the group A activators, these proteins require millimolar concentrations of Ca^{2+} for activity; they have virtually no activity in the absence of Ca^{2+}. Group C activators are serine proteases that require only Ca^{2+} and negatively charged phospholipids, but not Factor Va, for maximal activity. They have been purified and

Abbreviations used: berythractivase, **B. erythr***omelas* prothrombin **activase**; CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; FBS, fetal bovine serum; HUVECs, human umbilical-vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; MDC proteins, members of the snake venom metalloproteinase, disintegrin-like, cysteine-rich protein family; MoAb, monoclonal antibody; poly(A)$^+$, polyadenylated; S-2238, *H*-D-phenylalanyl-L-pipecolyl-L-argininine *p*-nitroanilide dihydrochloride; TFA, trifluoroacetic acid; UNIFESP-EPM, Universidade Federal de São Paulo-Escola Paulista de Medicina; VCAM-1, vascular cell adhesion molecule-1; vWF, von Willebrand factor.

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The nucleotide sequence of berythractivase has been deposited in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AF450503.

characterized from *Oxyuranus scutellatus* (coastal taipan) [12,13] and *Pseudonaja textiles* (eastern brown snake) venoms [14]. These activators cleave at both Arg^{271}-Thr^{272} and Arg^{320}-Ile^{321} bonds of prothrombin, converting it into mature thrombin [15]. Group D are serine proteases found in venoms of Australian elapids and their activities are strongly stimulated by Ca^{2+}, Factor Va and phospholipids [16–18]. They cleave prothrombin at two sites: Arg^{274}–Thr^{275} and Arg^{323}–Ile^{324} [19,20].

The physiological activation of prothrombin to the serine proteinase α-thrombin is catalysed by prothrombinase complex consisting of the serine proteinase, Factor Xa, cofactor Va and Ca^{2+}. Membranes containing anionic phospholipids are essential for the optimal function of this enzyme complex [21,22]. The conversion of prothrombin into thrombin requires the cleavage of two peptide bonds. The first cleavage occurs at the Arg^{320}–Ile^{321} bond, giving rise to the activated intermediate meizothrombin. A second cleavage at the Arg^{271}–Thr^{272} bond releases the fragment 1.2 and the serine proteinase α-thrombin [23]. In the absence of a phospholipidic surface, prothrombin can be activated by physiological concentrations of Factor Xa. However, the rate of activation is five orders of magnitude lower than the activation by prothrombinase complex [23], and the mechanism of cleavage proceeds through prethrombin-2 rather than through meizothrombin [24].

Snakebites from members of the genus *Bothrops* represent an important health problem. They are involved in more than 90 % of the human accidents from snakebites in South America. The venoms of most *Bothrops* species have been identified and possess direct prothrombin activation activity combined, or not, with thrombin-like and/or Factor X-activator activities. The coagulant action of some *Bothrops* venoms has been previously investigated, that of the venoms of *B. jararaca* (jararaca), *B. neuwiedi* and *B. atrox* being the most studied [9,10,25].

Bothrops erythromelas (jararaca-da-seca) snakes are widely distributed in north-eastern Brazil. Its venom presents a high level of haemorrhagic, coagulant and proteolytic activities [26–28]. This *Bothrops* species is particularly interesting because it does not present thrombin-like activity. Like other *Bothrops* species the coagulant activity of this venom was attributed to the remarkable presence of prothrombin and Factor X activators [26,28]. Up until now, however, the cloning and detailed biological properties of a prothrombin activator from *Bothrops* species have not been described. The present paper is the first report on the purification, characterization and molecular cloning of the prothrombin activator from *B. erythromelas* venom, which we name 'berythractivase'. The primary structure and characterization of berythractivase should improve the understanding of the structure–function and evolutionary relationship of snake-venom metalloproteinases.

MATERIALS AND METHODS

Venom and reagents

A pool of freeze-dried venom was collected from *B. erythromelas* adult female snakes from north-eastern Brazil. *o*-Phenanthroline, PMSF, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), human prothrombin, EDTA and benzamidine were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). *H*-D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride (S-2238) was obtained from Chromogenix AB (Mölndal, Sweden). Oligonucleotide primers for PCR, RPMI culture medium and fetal bovine serum (FBS) were from Life Technologies (Grand Island, NY, U.S.A.). Endothelial cell growth supplement from bovine neural tissue and fluorescein-conjugated mouse anti-human monoclonal antibodies (MoAbs) (IgG1), anti-(intracellular adhesion molecule-1) antibody [anti-ICAM-1 (CD 54)], anti-(vacular cell adhesion molecule) [anti-VCAM-1 (CD 106)] and control IgG1 were purchased from Calbiochem (La Jolla, CA, U.S.A.). The MoAb anti-E-selectin (CD 62E) and fluorescein-conjugated goat anti-mouse IgG $F(ab')_2$ were from Coulter Immunotech (Marseille, France). An enzyme immunoassay kit for von Willebrand factor (vWF) determination was from RDI-INC (Flanders, NJ, U.S.A.). All other chemicals used were of the highest purity grade available commercially.

Isolation of berythractivase

B. erythromelas venom (3.4 mg) was dissolved in 0.2 ml of 20 mM Tris/HCl buffer, pH 8.3, and the insoluble material was removed by centrifugation. The supernatant was applied directly to a cation-exchange Resource S column (Amersham Biosciences; 1 ml), equilibrated with the starting buffer (20 mM Tris/HCl, pH 8.3). Elution was achieved with a linear NaCl gradient from 0 to 0.5 M in the same buffer at a flow rate of 1.0 ml/min on a Pharmacia FPLC system. The A_{280} of the eluate was monitored. The eluted berythractivase was pooled, dialysed, concentrated and stored at −20 °C. Berythractivase from Resource S was submitted to reverse-phase chromatography, employing a C4 column (4.6 mm × 250 mm; J. T. Baker), the analytical HPLC system from Merck–Hitachi (model D-2500) and a Shimadzu UV monitor (model SPD-6AV) for protein detection at 214 nm. The solvents were 0.1 % trifluoroacetic acid (TFA) in water (Solvent A) and 0.1 % TFA/90 % acetonitrile (Solvent B). Berythractivase was applied on to the C4 column at a flow rate of 1.0 ml/min and eluted with a 35–50 % gradient of solvent B for 30 min. The protein concentration was estimated according to the method of Markwell et al. [29], or by its A_{280}.

Partial berythractivase amino acid sequence determination

The purified berythractivase (500 pM) was subjected to trypsin digestion (10 pM) in 100 mM Tris/HCl buffer, pH 8.0, containing 0.02 % $CaCl_2$ for 18 h at 37 °C. The reaction was interrupted using 15 % (v/v) formic acid. Fragments were separated by HPLC using a C4 column as described above. The peptides were eluted with a 0–100 % gradient of solvent B at a flow rate of 1.0 min/ml for 30 min. The N-terminal sequence and partial sequences from four internal peptides were determined by Edman degradation in an Applied BioSystems Microsequenator and searched against the SwissProt Database.

Prothrombin activating activity

The activation of prothrombin by berythractivase was indirectly determined by assaying thrombin formation from prothrombin, followed by assaying amidolytic activity towards a substrate, S-2238, specific for thrombin activity. The reaction was carried out in 50 mM Tris/HCl buffer (pH 8.3)/100 mM NaCl/44 nM prothrombin (Factor II)/60 mM berythractivase/40 mM S-2238 in a final volume of 500 μl. When cofactors (50 mM phospholipids and/or 50 mM $CaCl_2$) were present, they were included in the preincubation mixture. The hydrolysis of S-2238 by the thrombin formed was monitored spectrophotometrically at 405 nm for 5 min at 37 °C.

Effects of pH on the activity of berythractivase

The effect of different pH values on prothrombin activator was evaluated using various buffers and with prothrombin as a

substrate. Berythractivase was diluted and preincubated for 30 min at room temperature in the following buffer systems: 50 mM sodium citrate buffer (pH 3.0–6.0), 50 mM sodium phosphate buffer (pH 6.5–7.5) or 50 mM Tris/HCl buffer (pH 7.5–11.5). All buffers contained 100 mM NaCl. The residual activity was then measured as described above in the subsection 'Prothrombin activating activity'.

Determination of prothrombin fragments by berythractivase

The proteolytic activity of berythractivase towards prothrombin was assessed with 400 nM prothrombin incubated at 37 °C with 4 mM berythractivase in 50 mM Tris/HCl buffer, pH 8.0, containing 100 mM NaCl, to a final volume of 500 μl. Aliquots were removed at 0, 5, 10, 20, 30 and 60 min and analysed by non-reducing SDS/12 %-(w/v)-PAGE [30].

Inhibitors of berythractivase

Berythractivase (60 mM) was preincubated with either 20 mM PMSF dissolved in DMSO, 20 mM E-64, 20 mM benzamidine, 20 mM EDTA or 20 mM *o*-phenanthroline for 20 min at 37 °C prior to the addition of 44 nM prothrombin and 40 μM S-2238 to a final volume of 500 μl in 50 mM Tris/HCl buffer, pH 8.3, containing 100 mM NaCl.

Effect of berythractivase on fibrinogen

Human fibrinogen solution (2.0 mg/ml), diluted in 0.1 M sodium diethylbarbiturate, pH 7.75, containing 1.66 mM $CaCl_2$, 0.7 mM $MgCl_2$ and 92 mM NaCl, was preincubated at 37 °C with berythractivase (at a molar ratio, berythractivase/fibrinogen, of 1:50). Aliquots of 50 μl were removed at 0, 1, 5, 60, 120, 300 min and 24 h and subjected to reducing SDS/12 %-PAGE [30].

Fibrinolytic activity by the fibrin-plate method

Fibrinolytic activity was measured as described by Jespersen and Astrup [31]. Briefly, a fibrin–agarose gel was prepared by mixing human fibrinogen (2.0 mg/ml) with a preheated solution of 2.0 % agarose in 0.1 M sodium barbital buffer, pH 7.75, and 20 units/ml thrombin. Berythractivase (5 μg in 15 μl) was applied to the solidified gel and incubated overnight at 37 °C.

Cell cultures

Endothelial cells were obtained from human umbilical-cord veins (HUVECs) by collagenase digestion as described by Jaffe et al. [32]. HUVECs were identified by their cobblestone morphology and the binding of anti-(vWF) antibody. Cells were grown in RPMI 1640 medium supplemented with FBS (10 %, v/v), heparin (90 μg/ml), endothelial supplement growth factor (50 μg/ml), pyruvate (1 mM), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified 5 % CO_2 incubator. HUVECs used routinely for experiments were between the first and the third passage. To examine the effect of berythractivase, HUVECs were plated on 12-well dishes coated with 2 % gelatin and grown to confluence. In order to avoid the interference of heparin or growth factors, complete medium was removed and, after three washes, cells were incubated for 1 h in RPMI containing glutamine, antibiotics and different berythractivase concentrations. To exclude lipopolysaccharide interference, some experiments were performed in the presence of polymyxin B (7 μg/ml).

Expression of cell-adhesion molecules by flow cytometry

After berythractivase treatment of HUVECs, the medium was removed, the cells were washed and further cultured for 3 h in RPMI containing 10 % (v/v) FBS. Cells were then harvested by treatment with 0.25 % trypsin/0.02 % EDTA solution. After washing with Ca^{2+}/Mg^{2+}-free PBS containing 10 % FBS (PBS/FBS), the cells were resuspended in 50 μl of PBS/FBS containing saturating concentrations of FITC-conjugated anti-CD54, anti-CD106 or equivalent concentrations of an isotypic control IgG1. E-selectin was detected by staining cells with anti-CD62E MoAb for 1 h at 4 °C. The cells were then washed and incubated with goat anti-mouse IgG FITC-conjugated $F(ab')_2$ fragments (1:100). Cells were then fixed with 1 % paraformaldehyde, and analysed by flow cytometry in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA, U.S.A.). Appropriate settings of forward and side scatter gates were used to examine 10000 cells per experiment. The percentage of positive cells was determined by the thresholds set using isotypic controls. The numbers of fluorescent molecules per cell were indirectly measured by assessing the mean intensity of arbitrary units of fluorescence of cells.

vWF production

Release of vWF to the culture medium was measured quantitatively by an enzyme immunoassay. Microtitre plates were coated overnight at 4 °C with 100 μl of purified goat anti-(human vWF) antibody (93 μg/ml in 50 mM Na_2CO_3, pH 9.6). The remaining protein-binding sites were blocked with 150 μl of blocking solution. After washing, samples and standards diluted in Hepes/Tween/BSA solution were added and incubated for 120 min at 22 °C. Then a second anti-(vWF) antibody conjugated to horseradish peroxidase was added for another 90 min. Binding of the secondary antibody was detected by the addition of 100 μl of 2,2′-azinodi-(3-ethylbenzthiazoline sulphonate) and the absorbance was read at 405 nm. Results are expressed in ng/ml and they were estimated from a standard curve constructed from serial dilutions of normal pooled plasma, considering a vWF concentration of 10 μg/ml.

Haemorrhagic activity assay

The haemorrhagic activity was quantitatively determined by the method of Kondo et al. [33]. Berythractivase (15 μg) in NaCl (150 mM) was intradermally injected into the shaved dorsal skin of adult female Swiss mice. Crude venom (15 μg) with or without 20 mM EDTA was used as a control. After 24 h the animals were killed, the dorsal skin removed and the minimum haemorrhagic dose ('MHD') was defined as the amount of proteinase producing a spot with a diameter of 10 mm.

Statistics

All results were expressed as means $\pm$ S.E.M. Statistical analysis was performed by using Student's t test for paired data. Differences with a P value of < 0.05 were considered significant.

cDNA library construction

Briefly, total RNA from the venom glands of two adult specimens of *B. erythromelas* was extracted with Trizol reagent, according to the manufacturer (Life Technologies). Polyadenylated [poly(A)$^+$] RNA was purified from the total RNA preparation using an oligo(dT)–cellulose chromatography system (Life Technologies). Synthesis of cDNA was performed using the SuperScript Plasmid System, according to the procedure described by

the supplier (Life Technologies) and modified by Junqueira-de-Azevedo et al. [34]. Briefly, 6 μg of poly(A)$^+$ RNA was hybridized with a synthetic oligonucleotide dT_{18}–*Not*I primer-adapter (Amersham Pharmacia, Uppsala, Sweden) designed to prime the first strand for cDNA synthesis. Reverse transcription was achieved with SuperScript reverse transcriptase (Life Technologies). The second-strand synthesis was performed in the same reaction tube, in the presence of *Escherichia coli* RNase H, DNA polymerase I and ligase. The resulting double-stranded cDNAs were ligated to *Eco*RI adapters (Amersham Pharmacia) digested with *Not*I restriction enzyme, to allow their directionally cloning into the plasmid pGEM-11Zf(+) (Promega, Madison WI, U.S.A.) at *Eco*RI/*Not*I sites. The cDNA library was transformed using *E. coli* DH5-α cells, grown on 2YT broth [1.6% (w/v) tryptone/1% (w/v) yeast extract/0.5% (w/v) NaCl] containing 100 mg/ml ampicillin and the plasmids recovered after commercial (Life Technologies) plasmid preparation.

Berythractivase cDNA cloning

In order to obtain the cDNA encoding berythractivase, a sense degenerate primer was designed according to the N-terminal sequence (TPEQQ ...): 5′-CCGGATCCACTCCTGAACAAC-ARGC-3′ (primer 1). The SP6 primer complementary to SP6 promoter on pGEM11Zf(+) (Promega) was used as the antisense primer. PCR reactions were carried out in a PerkinElmer Thermal Cycler 9600 using *B. erythromelas* venom-gland cDNA library as template. A total of 30 cycles, consisting of a denaturation step at 94 °C for 1 min, an annealing step at 48 °C for 1 min and an extension step at 72 °C for 2 min, were performed. The PCR product was purified by 1.0%-agarose-gel electrophoresis and subcloned into the pGEM-T easy vector (Promega). The recombinant plasmids were sequenced by the dideoxy method using an ABI 377 Automated DNA Sequencer (Applied Biosystems) and the T7 and SP6 primers. The complete and correct berythractivase cDNA sequence, including the signal peptide and 5′-untranslated region was obtained by PCR and primer walking sequencing. The DNA sequence obtained, as well as the deduced amino acid sequence, were compared with the sequences in the GenBank® database using the BLAST Search Program [National Center for Biotechnology Information (NCBI), Bethesda, MD, U.S.A.] and the FASTA protein database.

RESULTS

Purification of berythractivase from *B. erythromelas*

The crude venom (3.4 mg) was chromatographed on Resource S ion-exchange column. Three major protein peaks were separated (Figure 1A). The first peak displayed fibrinolytic activity, the second one presented Factor X activating activity and the third peak presented strong prothrombin activating activity in the presence or absence of Ca^{2+} and phospholipids. In a single Resource S purification step about 0.17 mg of berythractivase was obtained. The purified protein from Resource S puification was highly homogeneous, as shown after analysis by C4 reversed-phase HPLC (Figure 1B). The homogeneity of purified berythractivase from the Resource S column was also verified by SDS/PAGE, showing a single protein band with a molecular mass of 78 kDa under reducing conditions (Figure 1C). The eluted protein from the C4 column was subjected to amino acid sequencing. The N-terminal sequence of berythractivase was determined up to 30 residues by Edman degradation, and internal peptide sequences were also obtained. These results gave amino acid sequence covering a total of 96 residues (shown underlined in Figure 6 below), which comprises 22.6% of the mature protein.

Prothrombin activating activity

Berythractivase readily activated prothrombin in a dose-dependent manner, independently of phospholipids and Ca^{2+} (Figure 2). In the first 1 min of incubation the hydrolysis of prothrombin by berythractivase was ten times higher than the hydrolysis of the prothrombin by total snake venom (results not shown). Berythractivase was not able to hydrolyse the S-2238, in contrast with thrombin (Figure 2). The activity of berythractivase was rapid and was completely inhibited by incubation with metal-chelating agents such as *o*-phenanthroline and EDTA. On the other hand, its activity was not inhibited by either PMSF, benzamidine, irreversible serine-proteinase inhibitors or by the cysteine proteinase inhibitor E-64. These results indicate that berythractivase differs remarkably from Factor Xa and that this enzyme is not a serine proteinase, but likely belongs to the metalloproteinase family of proteins. The structural and functional properties of the prothrombin activator from *B. erythromelas* are similar to those reported for the group A snake venom activator.

Effects of pH on the activity of berythractivase

The effect of pH on berythractivase activity was assayed using buffers with pH values ranging from 3.0 to 11.5. The results showed that berythractivase presented a maximum activity in the region of pH 8.0–9.0. Incubation of berythractivase at pH values below 6.0 or above 10.0 resulted in an abrupt decrease in prothrombin activating activity, the berythractivase being completely inactive at pH values below 4.0.

Determination of prothrombin fragments by berythractivase

The hydrolysis of prothrombin (72 kDa) by berythractivase under non-reducing conditions yielded fragments very similar to those described for activation of prothrombin by *B. atrox*, *E. carinatus* and *B. neuwiedi* group A venom activators [9,10]. Fragments of 72, 52, 36, 27 and 16 kDa were generated (Figure 3), most probably representing meizothrombin, F1/F2 activation peptide, α-thrombin, fragment-1 (F1) and fragment-2 (F2) respectively [35].

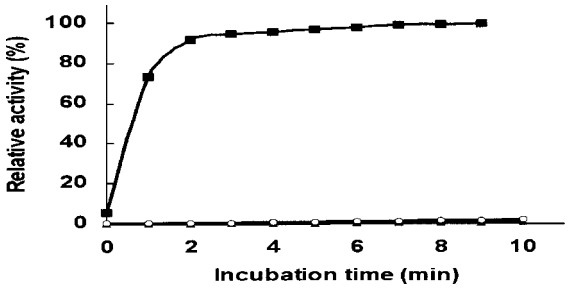

Figure 2 Prothrombin activation by berythractivase

Human prothrombin was incubated with berythractivase and S-2238 in Tris/HCl buffer. The amidolytic activity of prothrombin was monitored spectrophotometrically at 405 nm, in the course of 5 min, at 37 °C, and expressed as a percentage of maximal activity. ○, Prothrombin incubated with S-2238; ▲, berythractivase incubated with S-2238; ■, berythractivase incubated with prothrombin and S-2238.

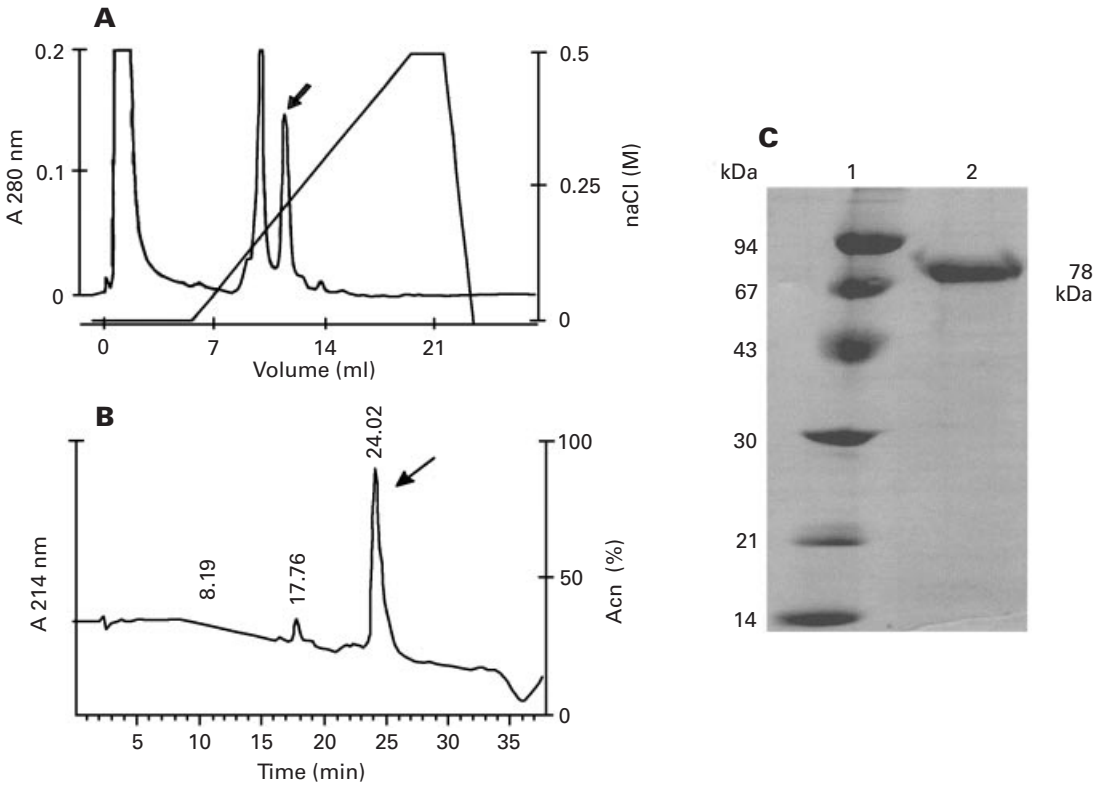

Figure 1 Purification of berythractivase

(**A**) Ion-exchange chromatography of *B. erythromelas* venom (3.4 mg) on a Resource S column equilibrated with 20 mM Tris/HCl buffer, pH 8.3 (1 cm) at 1 ml/min flow rate and 22 °C. Elution was performed with a linear gradient of 0–0.5 M NaCl and was monitored at 280 nm. The prothrombin activator is indicated by the arrow. One unit of specific activity is defined as the amount of activator which, activating prothrombin during 1 min, produced an amidolytic activity of 1 *A* unit/min, under fixed conditions defined in the method. (**B**) Reversed-phase HPLC of the active fraction (117 μg) on a C4 column equilibrated with solvent A (0.1% TFA in water) at 1 ml/min flow rate, for 30 min at 22 °C. The protein was eluted with 0–100% gradient of solvent B [acetonitrile (Acn)/solvent A, 9:1, v/v] and monitored at 214 nm. The major peak obtained after the C4 reversed-phase HPLC is indicated by an arrow. (**C**) SDS/PAGE under reducing conditions of purified berythractivase (20 μg) from the Resource S ion-exchange column. Lane 1, molecular-mass markers (phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovoalbumin, 43 kDa, carbonic anhydrase, 30 kDa; trypsin inhibitor, 21 kDa; α-lactoalbumin, 14.4 kDa); lane 2, berythractivase, 78 kDa.

Effect of berythractivase on fibrinogen and fibrin

The action of berythractivase was directed towards the Aα-chain of the fibrinogen molecule, without affecting the Bβ- or γ-chains. Concomitant with digestion of the Aα-chain was the appearance of fragments of approx. 44, 28 and 24 kDa (Figure 4). A small fragment of 16 kDa was evident after 5 h of incubation (300 min) and progressively increased as the concentration of larger fragments decreased. Berythractivase has a low fibrinolytic activity on fibrin plates, inducing lysis areas after a long period of incubation (results not shown).

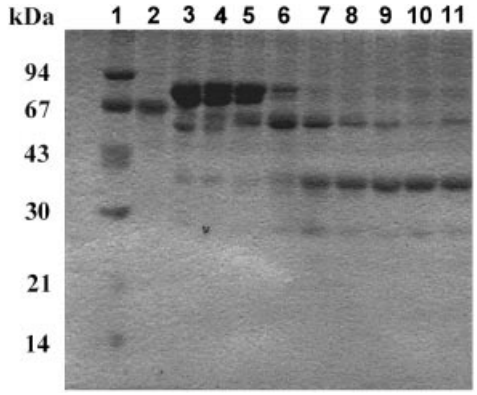

Figure 3 Time course of human prothrombin degradation by berythractivase

Prothrombin was activated at 37 °C in a reaction mixture containing berythractivase. At the time intervals indicated, aliquots of this reaction mixture were removed and analysed by SDS/PAGE (12% gel) under non-reducing conditions. Lane 1, molecular-mass markers; lane 2, berythractivase; lanes 3 and 4, prothrombin at the times 0 and 60 min; lanes 5–11, products of prothrombin hydrolyses at 0, 5, 10, 15, 20, 30 and 60 min respectively.

Endothelial-cell surface adhesion-molecule expression

Exposure of HUVECs to berythractivase induced both E-selectin and ICAM-1 expression without detectable increases in VCAM-1 (Figure 5A). Expression of ICAM-1 was also increased, but was not concentration-dependent under our assay conditions.

vWF release induced by berythractivase

Berythractivase induced vWF release after 1 h stimulation of HUVECs (Figure 5A). Preincubation of the purified protein with a polyclonal anti-bothropic serum completely inhibited berythractivase-induced vWF release (Figure 5B). Inhibition was not related to an unspecific effect of the serum or the immune complex formed, since the presence of serum did not modify

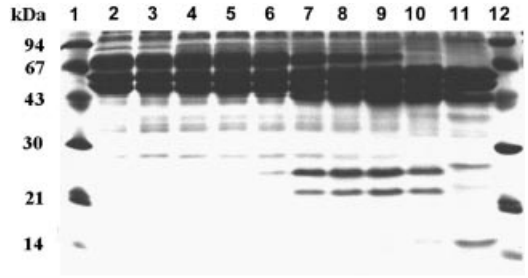

Figure 4 Fibrinogen hydrolysis by berythractivase

Human fibrinogen was incubated with berythractivase. Aliquots of 50 μl were removed at the times indicated and analysed by SDS/12%-PAGE under reducing conditions. Lanes 1 and 12, molecular-mass markers; lanes 2 and 3, human fibrinogen at times 0 and 60 min; lanes 4–11, products of fibrinogen hydrolysis at 0, 1, 5, 30, 60, 120, 300 min and 24 h respectively.

vWF release induced by thrombin (results not shown). vWF release by berythractivase was also not related to thrombin generation, since the presence of hirudin did not inhibit vWF release. As was observed for prothrombin activity, EDTA completely suppressed the ability of berythractivase to induce vWF release (Table 1). To determine the effect of berythractivase on vWF synthesis, HUVECs were first treated for 1 h with berythractivase. Then, after HUVEC washing, fresh medium was added and vWF levels were measured after 24 h. It was found that constitutive synthesis of vWF was similar in berythractivase and in control samples (results not shown).

Table 1 Inhibition of berythractivase-stimulated vWF release by EDTA

Berythractivase (Be; 250 μg/ml) was incubated with EDTA (20 mM) for 30 min at 37 °C before it was added to the cells for 1 h. Results are expressed as percentages of control and values are means ± S.E.M. for four independent experiments. The asterisks indicate $P < 0.05$ when compared with the control.

Treatment	vWF released (% of control)
Be (5 μg/ml)	270 ± 15*
+ EDTA (0.4 mM)	106 ± 4
Thrombin (1 unit/ml)	178 ± 7*
+ EDTA (0.4 mM)	180 ± 10*

Haemorrhagic activity

Berythractivase did not show haemorrhagic activity after 24 h of intradermal injection, in contrast with crude venom, which

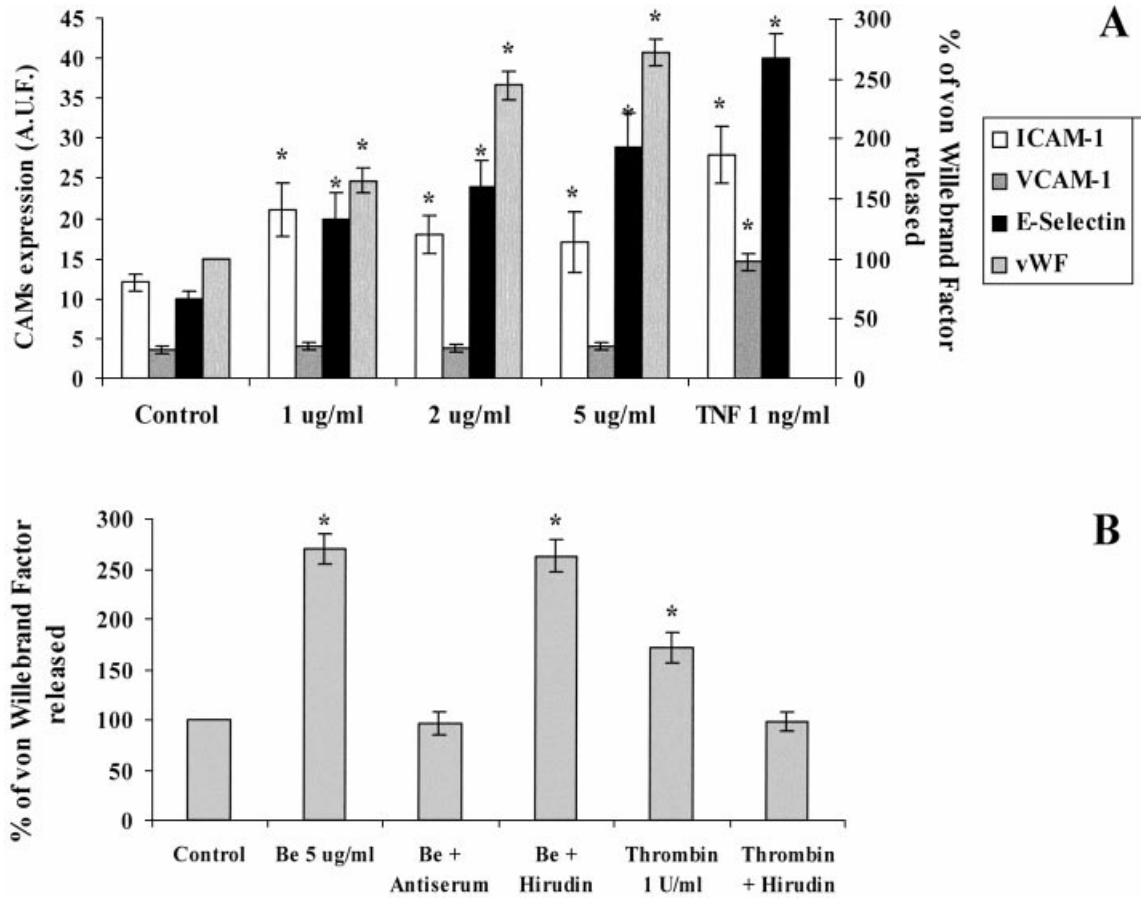

Figure 5 Cell-adhesion-molecule ('CAMs') expression and vWF release induced by berythractivase

(**A**) Endothelial cells were treated with berythractivase for 1 h at 37 °C. vWF was determined in culture supernatants and expressed as percentage of control. CAM expression was measured 4 h later by flow cytometry and expressed as arbitrary unit of fluorescence (A.U.F.). (**B**) Cells were incubated with berythractivase pretreated with anti-(bothropic venom) serum or in the presence of hirudin. Bars represent the means ± S.E.M. for four or five independent experiments. The asterisks indicate statistically significant differences between treatments and controls ($P < 0.05$).

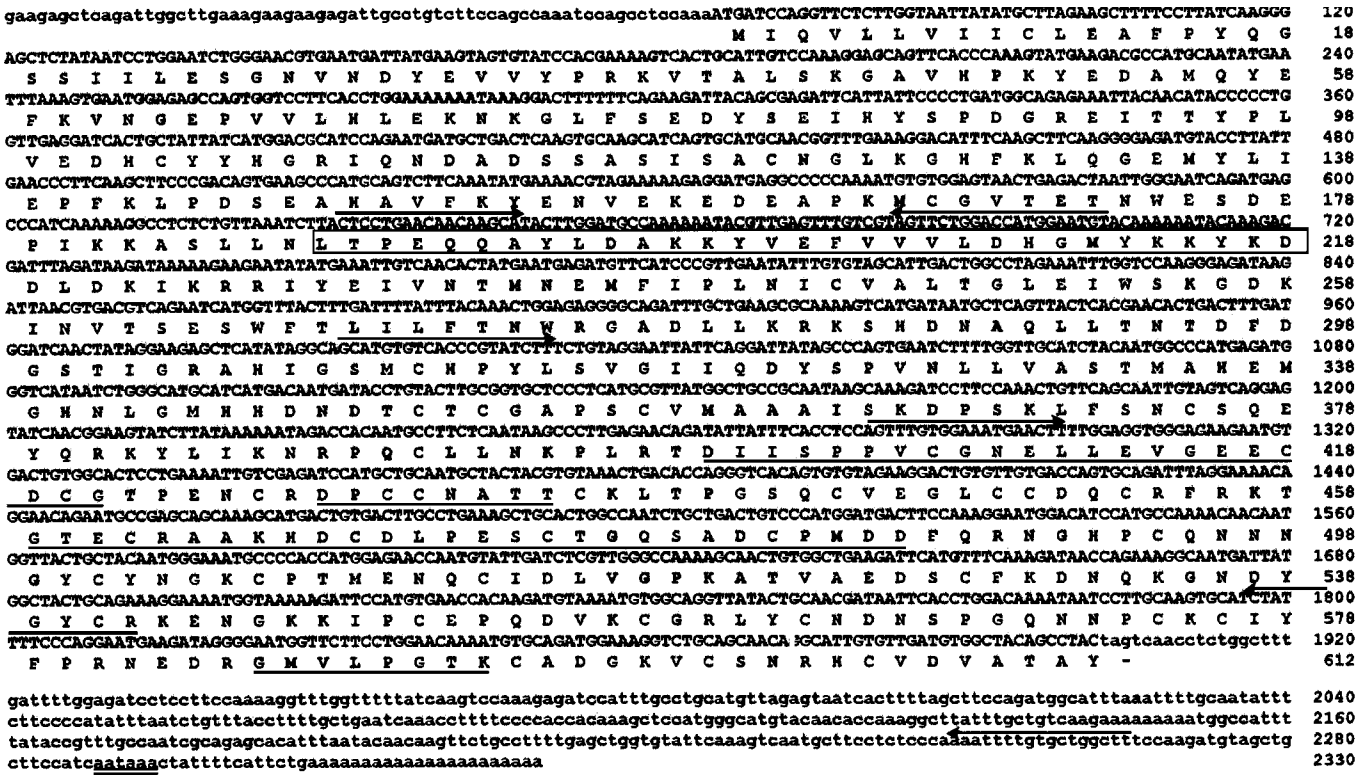

Figure 6 Nucleotide sequence of berythractivase cDNA and the deduced amino acid sequence

The Kozak sequence (TCCAAA) is located in the boxed region. The amino acid sequence derived from N-terminal sequence of berythractivase is boxed. The amino acid residues obtained by digestion of berythractivase and Edman reaction are underlined. 5′- and 3′-untranslated-region sequences are shown in lower-case letters. A polyadenylation signal (AATAAA) is double-underlined. Arrows indicate the position where the corresponding forward and reverse primers were designed for berythractivase cloning and primer walking sequencing.

showed significant haemorrhagic activity that was inhibited by the metal chelator EDTA.

Primary structure of berythractivase deduced by cDNA cloning

Screening the *B. erythromelas* venom-gland cDNA library by PCR with primers I and T7 resulted in the amplification of a DNA fragment of approx. 1700 bp. This fragment was cloned, twenty putative clones (Be-1–Be-20) were sequenced and seven clones were confirmed as positive clones. The Be-14 clone was chosen and completely sequenced. Using oligonucleotide primers based on this sequence and *B. erythromelas* cDNA library as template, the nucleotide sequence was extended in the 5′ direction by PCR. The nucleotide and predicted amino acid sequences of berythractivase cDNA are shown in Figure 6. The open reading frame encodes a precursor protein of 612 amino acids with a predicted molecular mass of 68.5 kDa. The start codon ATG is at nucleotide position 67 with the upstream sequence TCCAAA as the putative initiation site of translation just before the start codon [36]. The stop codon TAG was localized at nucleotides 1903–1905. The polyadenylation signal (AATAAA) was observed 14 nucleotides upstream from the poly(A) tail (Figure 6). The complete 2330 bp cDNA includes a 5′-untranslated region (66 bp) and a 3′-untranslated region (398 bp).

The deduced protein has the same structure of a multidomain preprometalloproteinase, which includes the conserved signal peptide for secretory proteins, a zinc metalloproteinase domain, the disintegrin-like domain and a cysteine-rich domain (Figure 7). The first 18 amino acids possess a hydrophobic core as a typical signal peptide with a cleavage motif for signal peptidase at Gly^{18}–Ser^{19} [37]. Cleavage at Asn^{187}–Leu^{188} yields the propeptide and the mature protein with an N-terminal sequence identical with that obtained from purified berythractivase. The metalloproteinase catalytic domain is composed of approx. 210 amino acids residues, the disintegrin-like domain is composed of 103 residues and the cysteine-rich domain of 114 residues (Figure 7).

The primary structure of berythractivase (Figure 7) resembled those from various metalloproteinases of the reprolysin family characterized from snake venoms. These proteins have functions as diverse as haemorrhagic factors, Factor X activators and prothrombin activators. The sequence similarity of mature berythractivase was highest with ACDL (77 %), an MDC haemorrhagic protein from *Agkistrodon contortrix laticinctus* (broad-banded copperhead) [38] and 69 % with VAP1, vascular-apoptosis-inducing protein 1 from *Crotalus atrox* (western diamondback rattlesnake) [39] (MDC proteins are members of the snake venom metalloproteinase, disintegrin-like, cysteine-rich protein family). The similarity between berythractivase precursor and precursor forms of RGD disintegrins is also high: 70 % with pro-catrocollastatin from *B. atrox* [40], 69 % with pro-jararhagin from *B. jararaca* [41] and 63 % with proecarin from *E. carinatus* [42] (Figure 7).

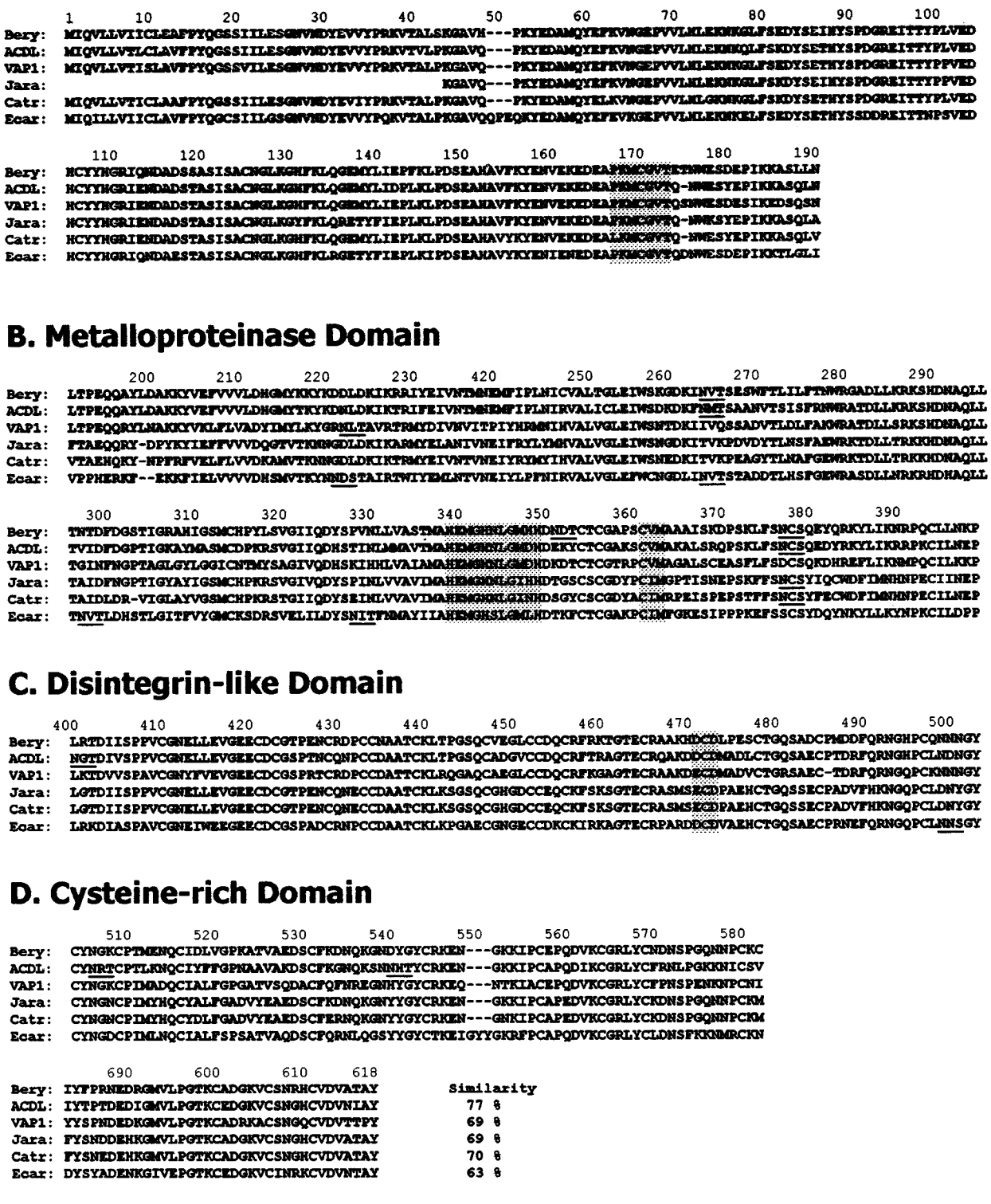

Figure 7 Alignment of the predicted amino acid sequence of berythractivase with members of the metalloproteinase-disintegrin protein (MDC) family

Sequences were obtained from the GenBank® database. Abbreviations: Bery, berythractivase (the present study); ACDL, *Agkistrodon contortrix laticinctus* metalloproteinase [36]; VAP-1, vascular apoptosis-inducing protein [37]; Catr, preprocatrocollastatin [38]; Jara, projararhagin [39]; and Ecar, ecarin [40]. Alignments are divided into four functional regions on the basis of sequence similarity. The 'cysteine switch' in (**A**), the putative zinc ligands and active site in (**B**) and the RGD-like sequence in (**C**) are highlighted with dotted shading. Putative N-glycosylation sites are underlined.

DISCUSSION

In the present study we have cloned the first prothrombin activator derived from venom of snakes of the genus *Bothrops*. The protein was purified by a single step and characterized as a single-chain protein of 78 kDa and it was named 'berythractivase' (***B. erythr**omelas* prothrombin **activase**). This procedure provides a method for the purification of homogeneous *Bothrops* snake-venom prothrombin activator that differs from the multi-step standard methods normally used [9,10].

SDS/PAGE analysis and the observation that the activity of berythractivase was not enhanced by the presence of phospholipids and/or Ca^{2+} indicated that it belongs to group A prothrombin activators [7].

A large number of metalloproteinases of different molecular masses and biological functions have been purified from snake venoms. Structural studies on these proteinases have suggested their organization into four classes, P-I –P-IV.

B. erythromelas venom-gland cDNA library screening resulted in amplification and cloning of a fragment with 1700 bp encoding a mature protein of 47.3 kDa. The molecular mass observed on SDS/PAGE (78 kDa; Figure 1C) is higher than that predicted (47.2 kDa) for the mature protein, probably because of the presence of N-glycosylation. Berythractivase presents three potential N-glycosylation sites in its amino acid sequence (Figure 6). Some snake-venom metalloproteinases have also been shown to be glycosylated [43,44]. Analysis of the cDNA sequence revealed that berythractivase is a member of the P-III class of the metalloproteinase protein family. The multidomain structure of berythractivase is in agreement with the common precursor model of snake-venom metalloproteinase/disintegrins [45].

After the cDNA sequencing of the first haemorrhagic metalloproteinases [41,46,47], it was shown that these enzymes are synthesized as zymogen precursors with a mechanism of latency similar to that observed with the matrix metalloproteinases [46]. The berythractivase prodomain is comprised of 188 amino acid residues that contain a 'cysteine switch' consensus sequence (PKMCGVT), 18 amino acid residues upstream from the proteinase domain (Figure 7). A similar cysteine-switch sequence was described in mammalian collagenases [48,49]. This sequence is involved in the generation of the active enzyme. In the latent form of the proenzyme, a free thiol group in this sequence is co-ordinated to the catalytic zinc ion at the active site. After cleavage and dissociation of the propeptide from the latent form of the proenzyme, it provides a free ligand of zinc, resulting in the activation of the proenzyme [50]. Given the similarity between the cysteine-switch consensus sequence of berythractivase and matrix metalloproteinases, a similar mechanism of latency and activation may be involved in the generation of mature active berythractivase.

The deduced sequence of berythractivase included the conserved consensus zinc-binding motif HEMGHNLGMHHD in the catalytic domain, indicating a relationship with the reprolysin subfamily of metalloproteinases [51]. Protein structures related to the reprolysin subfamily have also been identified in mammalian membrane-anchored reproductive proteins (fertilins), myogenetic proteins (meltins) and tumour necrosis factor-α convertase ('TACE') [51,52]. The zinc-binding motif is thought to be involved in the active site for the proteolytic activity of matrix metalloproteinases [53]. In the reprolysin subfamily there is another conserved consensus known as 'Met-turn' (CIMXP), which is also present in berythractivase. In this sequence, although methionine is always conserved, proline is not. On the other hand, the subsequent amino acid after methionine must be a hydrophobic residue such as leucine, isoleucine or alanine. This amino acid in berythractivase is alanine.

Disintegrins include a highly conserved RGD sequence, and they competitively block the adhesive functions of RGD-dependent integrins in various cell types [54]. In other MDC proteins, such as ecarin, atrolysine or jararhagin, RGD is replaced by ECD or DCD [55–57]. There is evidence indicating that the non-RGD disintegrin could inhibit platelet aggregation by binding to a different integrin receptor [40,58]. In its disintegrin-like domain, berythractivase has DCD. We have not yet investigated its role in platelet function. Berythractivase also have extended cysteine-rich C-terminus which is not present in the RGD disintegrins. The function of this domain is unclear, but it is also found in mammalian disintegrins.

It is known that *Bothrops* snake venoms induce local inflammatory lesions and disseminated intravascular coagulation [59]. Recently it was demonstrated that BaP-1 and BaH-1 [metalloproteinases from *Bothrops asper* (Central American lancehead) venom] could induce rat neutrophil chemotaxis and detachment of capillary endothelial cells respectively [59,60,61]. Although these findings suggest that endothelial cells may be intimately involved in the pathological responses induced by snake-venom metalloproteinases, to date there is no data establishing any effect of these proteinases on endothelial-cell activation.

Up-regulated expression of adhesion molecules on vascular endothelial cells is an initial event in leucocyte adhesion to the vascular wall, which is a crucial step in the process of inflammatory or immune response. Berythractivase increased surface expression of both E-selectin and ICAM-1 without modifiying VCAM-1. In this regard, berythractivase may behave differently from other modulators of cell adhesion molecules such as thrombin or tumour necrosis factor-α.

vWF is a multimeric plasma glycoprotein that mediates platelet adhesion to the subendothelium at the sites of vascular injury [62]. In addition, vWF binds to, and stabilizes, blood coagulation Factor VIII in the circulation [63] and is synthesized in endothelial cells and megakaryocytes [64,65]. Frequently it is used as a biochemical marker for endothelial-cell activation. vWF follows two pathways of secretion, namely a constitutive pathway directly linked to synthesis, and a regulated pathway, involving storage of mature molecules in endothelial-cell granules for release after stimulation by several agonists [66]. Our *in vitro* studies have shown that berythractivase was capable of modulating endothelial-cell release, but not vWF synthesis, in a concentration-dependent manner. Hirudin did not block vWF release, indicating that it was not associated with berythractivase-mediated thrombin generation. A direct effect of berythractivase on endothelial cells was confirmed by the complete suppression of vWF release by both a polyclonal anti-(bothropic venom) antiserum or EDTA treatment. Inhibition of berythractivase-induced vWF release by metal chelating agents indicated that the catalytic site of the protease is also involved in the triggering of endothelial-cell responses. Berythractivase-mediated vWF release could contribute to the disseminated intravascular coagulation that occurs after *Bothrops* snakebite.

Local and systemic haemorrhage is a common consequence of crotaline and viperine snakebites. Early studies using purified toxins have indicated that local haemorrhage can be attributed to metalloproteinases. Their effect seems to be related to the degradation of extracellular-matrix proteins [67]. It has been demonstrated that jararhagin (a metalloproteinase from *B. jararaca* venom) degrades vWF, which would also contribute to the haemorrhagic syndrome [55]. Berythractivase showed approx. 70% sequence similarity to jararhagin. However, preliminary

studies revealed that it did not degrade vWF (results not shown). In addition, berythractivase has a very low fibrinogenolytic activity (only the α-chain of fibrinogen was degraded after a longer period of incubation) without local haemorrhagic effects, in contrast with jararhagin. All these direct prothrombotic and proinflammatory endothelial-cell responses mediated by berythractivase may be involved in the local lesion and sytemic effects observed in humans bitten by *Bothrops*.

In conclusion, we characterized the first cDNA corresponding to a prothrombin activator metalloproteinase from a member of the snake genus *Bothrops*. Berythractivase is a prothrombin activator presenting a high structural similarity to other metalloproteinases, but it possesses distinct functional activities. Cumulative information about other snake-venom prothrombin activators is desirable and would provide structural comparative analyses that would enable us to understand the molecular basis underlying the functional differences among these highly structure-related metalloproteinases. It remains to be established whether different structural domains, with specific biological effects, are involved. In addition, the potential biotechnological application of berythractivase could be explored, the development of a prothrombin determination diagnostic kit being an example.

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