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cDNA Cloning and Characterization of Vascular Apoptosis-Inducing Protein 1

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Hemorrhagic snake venom induces apoptosis in vascular endothelial cells (VEC). In previous reports, we described the purification from crude venom of *Crotalus atrox* of two vascular apoptosis-inducing proteins (VAP1 and VAP2) that specifically induce apoptosis in vascular endothelial cells. We report here the cDNA cloning and characterization of VAP1. VAP1 cDNA encoded a protein with 610 amino acid residues. The amino acid sequence predicted from the cDNA indicated that VAP1 belongs to the metalloprotease/disintegrin family and that it is a multidomain polypeptide with a proprotein domain, a metalloprotease domain, a disintegrin-like domain, and a cysteine-rich domain. In the disintegrin-like domain, the sequence DECD replaces the RGD sequence that has frequently been found in such domains. We demonstrated that VAP1 has Zn^{2+}-dependent metalloprotease activity and degrades fibrinogen. After incubation in the presence of either EDTA or EGTA, VAP1 was hardly able to degrade fibrinogen and to induce apoptosis in VEC. Our results indicated that VAP1 is a new type of snake venom metalloprotease/disintegrin and suggest that the metalloprotease activity of VAP1 might be involved in the induction of apoptosis by VAP1 in VEC. © 2000 Academic Press

Key Words: **snake venom; apoptosis; vascular endothelial cells; metalloprotease/disintegrin family.**

Vascular endothelial cells (VEC) play important roles in blood clotting, vascular contraction, and the control of vascular permeability (1). VEC are also important in wound healing, in the progression of cancer, in the homeostatic maintenance of the circulatory system, in embryogenesis and in embryonic growth because of their capacity for angiogenesis (1, 2). Degradation of blood vessels is thought to be as important as angiogenesis in such phenomena. For example, degradation of blood vessels in tumors induces tumor regression (3) and degradation of blood vessels during the healing of a wound is important for prevention of a hypertrophic scar (4). Furthermore, there are many vascular degenerative diseases, such as atherosclerosis and diabetic angiopathy, as well as hemorrhagic diseases or conditions that involve vascular degradation and are caused by certain viruses, bacteria, snake venoms, and autoantigens (5), but the mechanisms of vascular degradation remain to be defined. In nervous and immune systems, it has been demonstrated that apoptotic cell death is involved in various degenerative phenomena. In vascular systems, apoptotic cell death has been reported in the degeneration of internal organs (6).

We have investigated cell death in VEC because the evidence suggests that such cell death is likely to play an active role in the control of vascular degeneration (7, 8). In previous reports, we described the purification and characterization of two vascular apoptosis-inducing proteins, VAP1 and VAP2, from the hemorrhagic venom of the snake *Crotalus atrox* (9, 10). VAP1 was purified as a homodimeric protein with a molecular mass of 110 kDa and an isoelectric point of 8.5. VAP2 was purified as a monomeric protein with a molecular mass of 55 kDa and an isoelectric point of 4.5. The apoptotic activities of both VAPs were specific to VEC. Analysis of the partial amino acid sequences of

The sequence reported in this paper have been submitted to the DDBJ/EMBL/GenBank database (Accession No. AB042840).

Abbreviations used: VAP, vascular apoptosis-inducing protein; MCS, multiple cloning site; VEC, vascular endothelial cells; FGF, fibroblast growth factor; LAO, L-amino acid oxidase; PVDF, polyvinylidene difluoride; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS^-, phosphate-buffered saline without magnesium and calcium; PMSF, phenylmethylsulfonyl fluoride.

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VAP1 and VAP2 revealed that both VAPs resembled members of the metalloprotease/disintegrin family.

Snake venoms are complex mixtures of many biologically active polypeptides. Some of these polypeptides are members of the metalloprotease/disintegrin family, and various activities, such as those of hemorrhagic metalloprotease (11, 12), fibrinogenase (13), prothrombin activator (14), platelet aggregation inhibitor (15), have been detected in snake venoms. It is likely that similar domain structures, such as a proprotein domain, a metalloprotease domain, a disintegrin-like domain, and a cysteine-rich domain, are conserved in the protein precursors of the members of this family. Snake venoms are also a rich source of L-amino acid oxidase (LAO) (16, 17). LAO-induced cell death has been observed in a variety of cell lines, such as leukemia cells and carcinoma cells. By contrast, VAP1 and VAP2 only induced apoptosis in VEC and all other cell lines tested were resistant to the apoptosis-inducing effect of these VAPs (10).

In this study, we cloned a cDNA for VAP1 in order to characterize the protein and compare it to other members of the metalloprotease/disintegrin family and cytotoxic factors in snake venom.

MATERIALS AND METHODS

Reagents. MCDB-107 medium for cell culture was purchased from Kyokuto Pharmaceutical Industries (Tokyo, Japan). Fibroblast growth factor (FGF) was extracted from bovine brain as described by Lobb and Fett (18). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY). Crude snake venom was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of ultrapure grade.

Purification of vascular apoptosis-inducing protein 1. VAP1 was purified from the crude venom of *Crotalus atrox* as described previously (9). In brief, crude venom was fractionated by column chromatography on hydroxyapatite and by isoelectric focusing on a polyacrylamide gel. The activity in each fraction was examined by monitoring the viability of cultured VEC and fractions with apoptotic activity were identified.

Determination of partial amino acid sequences. After SDS–PAGE (10% polyacrylamide), the band corresponding to VAP1 was excised from the gel and the protein was digested by cyanogen bromide or with trypsin. The digests were fractionated by SDS–PAGE (15% polyacrylamide) and peptide fragments were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Bedford, MA). Amino acid sequences of peptide fragments were determined with a protein sequencer (PSQ-1; Shimazu Instrument Inc., Japan).

The cDNA library and primers. A ZAP cDNA library was previously prepared from the venom glands of *Crotalus atrox* (19). All primers were purchased from KURABO (Osaka, Japan).

Cloning and sequencing of cDNA. The amino acid sequences of peptides derived from purified VAP1 were used to design degenerate sense and antisense primers as indicated in Fig. 1. The polymerase chain reaction (PCR) was performed in a final volume of 100 μl. The reaction mixture contained 200 pmol of each primer, 0.1 μg of the abovementioned cDNA library as template, 0.2 mM each dNTP and 2.5 units of *TaKaRa EX Taq* DNA polymerase (TaKaRa). After an initial denaturation step at 94°C for 90 s, amplification was allowed to proceed for 35 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 74°C. The final extension step was allowed to proceed at 74°C for 7 min. The products of PCR were fractionated by agarose gel electrophoresis. Bands of DNA fragments were excised from the gel and DNA fragments were subcloned into the pCR2.1 TOPO vector using a TOPO TA Cloning Kit (Invitrogen).

Sequences of subcloned fragments were determined with both an ABI PRISM Dye Terminator Cycle Sequencing Kit and a Dye Primer Cycle Sequencing Kit (Perkin-Elmer). The internal sequence of VAP1 cDNA was extended by PCR cloning using the cDNA library prepared from *Crotalus atrox*. Specific sense (5′-GTAAACTGAGACAAGGAGC-3′) and antisense (5′-CTTCTCCCACCTCCACAAAG-3′) primers, corresponding to the middle portion of the clone obtained after the first amplification, were prepared, as well as Z-primers, which corresponded to the multiple cloning site (MCS) of the ZAP vector (Z-sense, 5′-AAGGGAACAAAAGCTGGAG-3′; Z-antisense, 5′-GTAATACGACTCACTATAGGG-3′). PCR cloning was then performed with template DNA, specific primers and Z-primers. The products of PCR were fractionated by agarose gel electrophoresis. Bands of DNA fragments were excised from the gel and DNA fragments were subcloned into pCR2.1 TOPO and sequenced.

Proteolytic activity. The proteolytic activity of VAP1 was assessed with fibrinogen as the substrate. The reaction mixture contained 10 mM Tris–HCl (pH 7.5), 1 mM $CaCl_2$, VAP1 and fibrinogen. After incubation for 24 h at 37°C, samples were fractionated by SDS–PAGE (12% polyacrylamide) and proteins were stained with Coomassie brilliant blue.

Cell cultures. Human umbilical vein endothelial cells were obtained as described by Jaffe *et al.* (20). The cells were cultured on gelatin-coated plastic dishes in MCDB-107 culture medium that had been supplemented with 10% FBS, 70 ng/ml FGF, and 100 μg/ml heparin at 37°C in an atmosphere of 5% carbon dioxide and 95% air. The cells were stained immunochemically by treatment with antibodies raised in rabbit against human factor VIII to confirm that they were endothelial cells.

Induction of apoptosis by VAP1. VEC were grown until cultures reached confluence. The medium was replaced with basal medium (MCDB-107 without FGF and FBS) after cells had been washed once with phosphate-buffered saline without magnesium and calcium (PBS^-). VAP1 was incubated in 10 mM sodium phosphate buffer (pH 7.0) with 0.01% SDS for 3 h at 37°C. The incubation was done in the presence or absence of either 2 mM EDTA or 2 mM EGTA. Cells were incubated with or without above-mentioned VAP1 (1 μg/ml). After treatment for 18 h, cells were photographed under a light microscope.

Nuclear-fragmentation assay. VEC were grown until cultures reached confluence. The medium was replaced with basal medium (MCDB-107 without FGF and FBS) after cells had been washed once with phosphate-buffered saline without magnesium and calcium (PBS^-). VAP1 was incubated in 10 mM sodium phosphate buffer (pH 7.0) with 0.01% SDS for 3 h at 37°C. The incubation was done in the presence or absence of either 2 mM EDTA or 2 mM EGTA. Cells were

FIG. 1. PCR primers and nucleotide sequence of the VAP1 cDNA. (A) Oligonucleotide primers derived from peptides in digests of purified VAP1. Primer 1 is a sense primer and primer 2 is an antisense primer; (B) Nucleotide sequence of VAP1 cDNA and the deduced amino acid sequence, which is shown below the nucleotide sequence. A potential site of N-linked glycosylation is indicated by a black dot. The peptide sequences obtained by digestion of VAP1 are underlined.

A

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                D  H  R  E  F  L  I  K

Primer 1   5' GAT CAT CGT GAA TTT TTT AT  3'
             C   C A C   G   C C C
                   A           A
                   G           G


                M  A  D  Q  C  I  A  L  F  G  P  G  A

Primer 2   3' TAC CGT CTA GTT ACA TAT CG  5'
                 C  G  C  G  A
                 A           G
                 G
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B

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          GGCACGAGCAACAGAGGAAGAGCTCAGATTGGCTTGAAAGCAGGAAGAGATTGCCTGTCTTCCAGCCAAATCCAGCCTCCAAA   -1

ATGATCCAGGTTCTCTTGGTAACTATATCATTAGCAGTTTTTCCTTATCAAGGGAGCTCTGTAATCCTGGAATCTGGGAACGTGAATGAT    90
M  I  Q  V  L  L  V  T  I  S  L  A  V  F  P  Y  Q  G  S  S  V  I  L  E  S  G  N  V  N  D

TATGAAGTAGTGTATCCACGAAAAGTTACTGCATTGCCCAAAGGAGCAGTTCAGCCAAAGTATGAAGACGCCATGCAATATGAATTTAAG   180
Y  E  V  V  Y  P  R  K  V  T  A  L  P  K  G  A  V  Q  P  K  Y  E  D  A  M  Q  Y  E  F  K

GTGAATGGAGAGCCAGTGGTCCTTCACCTGGAAAAAAATAAAGGACTTTTTTCAGAAGATTACAGTGAGACTCATTATTCCCCTGATGGC   270
V  N  G  E  P  V  V  L  H  L  E  K  N  K  G  L  F  S  E  D  Y  S  E  T  H  Y  S  P  D  G

AGAGAAATTACAACATACCCCCCGGTTGAGGATCACTGCTATTATCATGGACGCATCGAGAATGATGCTGACTCAACTGCAAGCATCAGT   360
R  E  I  T  T  Y  P  P  V  E  D  H  C  Y  Y  H  G  R  I  E  N  D  A  D  S  T  A  S  I  S
                                                                    proprotein domain

GCATGCAACGGTTTGAAAGGACATTTCAAGCTTCAAGGGGAGATGTACCTTATTGAACCATTGAAGCTTCCCGACAGTGAAGCTCATGCA   450
A  C  N  G  L  K  G  H  F  K  L  Q  G  E  M  Y  L  I  E  P  L  K  L  P  D  S  E  A  H  A

GTCTTCAAATATGAAAATGTAGAAAAAGAGGATGAGGCCCCCAAAATGTGTGGGGTAACCCAGAATTGGGAATCATATGAGCCCATCAAA   540
V  F  K  Y  E  N  V  E  K  E  D  E  A  P  K  M  C  G  V  T  Q  N  W  E  S  Y  E  P  I  K

AAGGCCTCTCAGTCAAATCTTACTCCTGAACAACAAAGATACTTGAACGCCAAAAAATACGTGAAGCTTTTCCTAGTTGCGGACTACATA   630
K  A  S  Q  S  N  L  T  P  E  Q  Q  R  Y  L  N  A  K  K  Y  V  K  L  F  L  V  A  D  Y  I

ATGTACTTGAAATATGGCCGCAATTTAACTGCTGTAAGAACAAGAATGTATGATATTGTCAACGTTATAACTCCGATTTACCATCGTATG   720
M  Y  L  K  Y  G  R  N  L  T  A  V  R  T  R  M  Y  D  I  V  N  V  I  T  P  I  Y  H  R  M
                                 •

AATATTCATGTAGCACTGGTTGGCCTAGAAATTTGGTCCAACACAGATAAAATTATCGTGCAGTCATCAGCAGATGTTACTTTGGACTTA   810
N  I  H  V  A  L  V  G  L  E  I  W  S  N  T  D  K  I  I  V  Q  S  S  A  D  V  T  L  D  L

TTTGCAAAGTGGAGAGCGACAGATTTGCTGAGCCGCAAAAGTCATGATAATGCTCAGTTACTCACGGGCATTAACTTCAATGGACCAACT   900
F  A  K  W  R  A  T  D  L  L  S  R  K  S  H  D  N  A  Q  L  L  T  G  I  N  F  N  G  P  T
                                                                    metalloprotease domain

GCAGGACTTGGTTACTTGGGCGGCATATGCAATACGATGTATTCTGCAGGAATTGTTCAGGATCATAGCAAAATACATCATTTGGTTGCA   990
A  G  L  G  Y  L  G  G  I  C  N  T  M  Y  S  A  G  I  V  Q  D  H  S  K  I  H  H  L  V  A

ATTGCAATGGCCCATGAGATGGGTCATAATCTGGGCATGGATCATGACAAAGATACCTGTACTTGTGGGACTAGGCCATGTGTTATGGCT  1080
I  A  M  A  H  E  M  G  H  N  L  G  M  D  H  D  K  D  T  C  T  C  G  T  R  P  C  V  M  A

GGGGCACTAAGCTGTGAAGCTTCCTTTCTGTTCAGCGATTGTAGTCAGAAAGATCATCGGGAGTTTCTTATTAAAAACATGCCTCAATGC  1170
G  A  L  S  C  E  A  S  F  L  F  S  D  C  S  Q  K  D  H  R  E  F  L  I  K  N  M  P  Q  C

ATTCTTAAGAAACCCTTGAAAACAGATGTTGTTTCACCTGCAGTTTGTGGAAATTACTTTGTGGAGGTGGGAGAAGAATGTGACTGTGGC  1260
I  L  K  K  P  L  K  T  D  V  V  S  P  A  V  C  G  N  Y  F  V  E  V  G  E  E  C  D  C  G

TCTCCTAGAACTTGTCGAGATCCATGCTGTGATGCTACTACGTGTAAACTGAGACAAGGAGCACAGTGTGCAGAAGGACTGTGTTGTGAC  1350
S  P  R  T  C  R  D  P  C  C  D  A  T  T  C  K  L  R  Q  G  A  Q  C  A  E  G  L  C  C  D
                                                                    disintegrin-like domain

CAATGCAGATTTAAGGGAGCAGGAACAGAATGCCGGGCAGCAAAGGATGAGTGTGACATGGCTGATGTCTGCACTGGCCGATCTGCGGAG  1440
Q  C  R  F  K  G  A  G  T  E  C  R  A  A  K  D  E  C  D  M  A  D  V  C  T  G  R  S  A  E

TGTACAGATCGCTTCCAAAGGAATGGACAACCATGCAAAAACAACAACGGTTACTGCTACAATGGGAAATGCCCCATCATGGCAGACCAA  1530
C  T  D  R  F  Q  R  N  G  Q  P  C  K  N  N  N  N  G  Y  C  Y  N  G  K  C  P  I  M  A  D  Q

TGTATTGCTCTCTTTGGGCCAGGTGCAACTGTGTCTCAAGATGCATGTTTTCAGTTTAATCGTGAGGGCAATCATTATGGCTACTGCAGA  1620
C  I  A  L  F  G  P  G  A  T  V  S  Q  D  A  C  F  Q  F  N  R  E  G  N  H  Y  G  Y  C  R

AAGGAACAAAATACAAAAATTGCATGTGAACCACAAGATGTAAAATGTGGCAGGTTATACTGCTTCCCTAATTCACCTGAAAACAAGAAT  1710
K  E  Q  N  T  K  I  A  C  E  P  Q  D  V  K  C  G  R  L  Y  C  F  P  N  S  P  E  N  K  N
                                                                    cysteine-rich domain

CCTTGCAATATCTACTATTCACCCAATGATGAAGATAAGGGAATGGTTCTTCCTGGAACTAAATGTGCAGATAGAAAGGCCTGCAGCAAC  1800
P  C  N  I  Y  Y  S  P  N  D  E  D  K  G  M  V  L  P  G  T  K  C  A  D  R  K  A  C  S  N

GGGCAATGTGTTGATGTGACTACACCCTACTAATCAACCTCTGGCTTCTCTCAGATTTGATTTTGGAGATCCTTCTTCCAGAAGGTTCAA  1890
G  Q  C  V  D  V  T  T  P  Y  *

CTTCCCTCAAGTCCAAAGAGACCCATCTGCCTGCATCCTACTAGTAAATCACCCTTAGCTTTCATATGGAATCTAAATTCTGCAATATTT  1980
CTTCTCCATATTTAATCTGTTTACCTTTTGCTGTAATCAAACTTTTTCCCGTCACAAAGCTCCATGGGCATGTACAACACCAACGACTTA  2070
TTTGCTGTCAAAAAAAAAAAAAAAAAA
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incubated with or without above-mentioned VAP1 (1 μg/ml) for 12 h. Then they were trypsinized and washed once with PBS^- and fixed in 1% glutaraldehyde for 6 h at room temperature. Each suspension of cells was centrifuged and cells were suspended in PBS^- and stained with 10 μM Hoechst 33258 (Sigma). One drop of stained cells was placed on a glass slide and nuclear fragmentation was examined by fluorescence microscopy.

RESULTS

Partial amino acid sequences and cDNA cloning of VAP1. After SDS–PAGE of partially purified VAP1, the band corresponding to VAP1 was excised from the gel. The purified VAP1 was treated with cyanogen bromide or trypsin and the resultant peptides were separated by SDS–PAGE and blotted onto a PVDF membrane. Then the amino acid sequences of the peptides were determined (Fig. 1A). We selected two peptide sequences for the design of degenerate primers for PCR (Fig. 1A). Amplification with primer 1 and primer 2 yielded a product of 400 bp. Using oligonucleotide primers based on this sequence and the *Crotalus atrox* cDNA library, we extended this sequence in the 5′ and 3′ directions. The 5′-extended clone included a putative site for the initiation of translation and the 3′-extended clone included a putative termination codon. The nucleotide and predicted amino acid sequences of the open reading frame are shown in Fig. 1B. The open reading frame includes an initiation codon for methionine and a termination codon, TAA. There is a termination codon upstream of the initiation codon for methionine. The predicted amino acid sequence contains all four internal peptides that we obtained by treating VAP1 with cyanogen bromide or trypsin.

The open reading frame encoded a protein of 610 amino acids with a predicted molecular mass of 67 kDa. In the amino-terminal sequence, we found a typical motif for cleavage by a signal peptidase, namely, Gly18-Ser19. The putative signal peptide was followed by a proprotein domain, a metalloprotease domain, a disintegrin-like domain, and a cysteine-rich domain (Fig. 2). The predicted amino acid sequence was analyzed for similarities to other sequences with the FASTA program. VAP1 was 72% homologous to extracellular matrix metalloprotease, a metalloprotease/disintegrin-like protein of *Agkistrodon contortrix laticinctus;* 62% homologous to ecarin, a prothrombin activator of *Echis carinatus;* and 58% homologous to Ht-a, a hemorrhagic metalloprotease of *Crotalus atrox.* EAP-1, MS2 and meltrin α, which belong to the ADAM family, were approximately 39, 38, and 35% homologous to the deduced sequence of VAP1, respectively.

Hydrolysis of fibrinogen by VAP1. We found that the consensus sequence of a zinc-binding site, H-E-X-X-H-X-X-G-X-X-H, was conserved in the metalloprotease domain of VAP1. A zinc ion is likely to be coordinated by histidine residues and to act as a catalytic base. We examined VAP1 to determine whether it might have proteolytic activity, using fibrinogen as the substrate. Fibrinogen (100 μg) was hydrolyzed during incubation with VAP1 (0.5 μg) (Fig. 3). However, the hydrolysis of fibrinogen was strongly inhibited after VAP1 had been treated first with 4 mM EDTA or 4 mM EGTA. The upper band in lane 1 appears to become thinner in lane 3 and 4 as well as in lane 2. However, in the presence of EDTA or EGTA, the band become thinner (data not shown), probably due to loss of microheterogeneity. Indeed, the bands corresponding to the fragment, that denote the proteolytic activity of VAP1 in lane 2, were not detectable in lanes 3 and 4. Thus, the hydrolysis of fibrinogen appeared to have been due to the metalloprotease activity of VAP1.

The proteolytic activity of VAP1 is involved in apoptosis in VEC. When we treated VEC with VAP1, morphological changes occurred that were typical of apoptosis, namely, cell shrinkage and the formation of blebs on the cell surface that resulted in the generation of apoptotic bodies (Fig. 4B; left). By contrast, when VAP1 was incubated with 2 mM EDTA or 2 mM EGTA prior to its addition to cell cultures, no such morphological changes were observed (Figs. 4C and 4D; left). The definitive biochemical hallmark of apoptosis in many types of cell is the condensation of nuclear chromatin. We fixed VEC in 1% glutaraldehyde with or without prior exposure of the cells to VAP1 and then we examined the cells after staining them with Hoechst 33258. After treatment of VEC with VAP1 for 12 h, condensation of chromatin was clearly detectable (Fig. 4B; right). When VEC were treated with VAP1 that had previously been incubated with 2 mM EDTA or 2 mM EGTA, no condensation of chromatin was observed (Figs. 4C and 4D; right).

DISCUSSION

We previously reported the purification of two vascular apoptosis-inducing proteins, VAP1 and VAP2,

FIG. 2. Comparison of the deduced amino acid sequence of VAP1 with those of members of the metalloprotease/disintegrin family. (A) Metalloprotease domain; (B) disintegrin-like domain. The consensus sequence of the zinc-binding site is underlined. The DECD sequence of VAP1 and the corresponding sequences in the other proteins are shown in white print on a black background. The numbering of residues corresponds to that of VAP1. The alignments were generated with the Clustal W program. Abbreviations are as follows; agkis, metalloprotease/disintegrin-like protein of *Agkistrodon contortrix laticinctus;* ecarin, precursor to ecarin, prothrombin activator of *Echis carinatus;* ht-a, hemorrhagic toxin a of *Crotalus atrox;* macaeap1, precursor to epididymal apical protein 1 of *Macaca fascicularis;* musms2, precursor to the cell surface antigen MS2 of *Mus musculus;* musmela, meltrin α, a metalloprotease/disintegrin of *Mus musculus.*

A Metalloproteinase domain

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                                                                    249
VAP1            EQQRYLNAKKYVKLFLVADYIMYLKYGRNLTAVRTRMYDIVNVITPIYHRMNIHVALVGL
agkis           EQQAYLDAKKYVEFVVVLDHGMYTKYKDNLDKIKTRIFEIVNTMNEMFIPLNIRVALICL
ecarin          HER--KFEKKFIELVVVVDHSMVTKYNNDSTAIRTWIYEMLNTVNEIYLPFNIRVALVGL
ht-a            ----ERLTKRYVELVIVADHRMFTKYNGNLKKIRKWIYQIVNTINEIYIPLNIRVALVRL
macaeap1        PKMKAIHNEKYIELFIVADDTVYRRNSHPHNKLRNRIWGMVNFVNMIYKTLNIHVTLVGI
musms2          RNWLIPRETRYVELYVVADSQEFQKLG-SREAVRQRVLEVVNHVDKLYQELSFRVVLVGL
musmela         HKRETLKMTKYVELVIVADNREFQRQGKDLEKVKQRLIEIANHVDKFYRPLNIRIVLVGV

                                                                    309
VAP1            EIWSNTDKIIVQSSADVTLDLFAKWRATDLLSRKSHDNAQLLTGINFNGPTAGLGYLGGI
agkis           EIWSDKDKFNMTSAANVTSISFRNWRATDLLKRKSHDNAQLLTVIDFDGPTIGKAYMASM
ecarin          EFWCNGDLINVTSTADDTLHSFGEWRASDLLNRKRHDHAQLLTNVTLDHSTLGITFVYGM
ht-a            EIWSNGDLIDVTSAANVTLKSFGNWRVTNLLRRKSHDNAQLLTAIDLDEETLGLAPLGTM
macaeap1        EIWTHEDKIELHSNIETTLLRFSSWQERILKTRKDFDHVVLLSGKWIYTHVQGISYPAGM
musms2          EIWN-KDKFYISRYANVTLENFLSWREQNLQGQHPHDNVQLITGVDFIGSTVGLAKVSAL
musmela         EVWNDIDKCSISQDPFTRLHEFLDWRKIKLLPRKSHDNAQLISGVYFQGTTIGMAPIMSM

                                                                    363
VAP1            CNTMYSAGIVQDHSKIHHLVAIAMAHEMGHNLGMDHDKDT--CTCGTRP----CVMAGAL
agkis           CDPKRSVGIIQDHSTINLMMAVTMAHEMGHNLGMDHDEKY--CTCGAKS----CVMAKAL
ecarin          CKSDRSVELILDYSNITFNMAYIIAHEMGHSLGMLHDTKF--CTCGAKP----CIMFGKE
ht-a            CDPKLSIGIVQDHSPINLLVAVTMAHELGHNLGMVHDENR--CHCSTPA----CVMCAVL
macaeap1        CLPYYSTSIIKDLLPDTNIIANRMAHQLGHNLGMQHDEFP--CTCPSGK----CVMDSDG
musms2          CS-RHSGAVNQDHSKNSIGVASTMAHELGHNLGMSHDEDIPGCYCPEPREGGGCIMTESI
musmela         CTAEQSGGVVMDHSDSPLGAAVTLAHELGHNFGMNHDTLERGCSCRMAAEKGGCIMNPST

                                                        396
VAP1            SCEASFLFSDCSQKDHREFLIKNMPQCILKKP
agkis           SRQPSKLFSNCSQEDYRKYLIKRRPKCILNEP
ecarin          SIPPPKEFSSCSYDQYNKYLLKYNPKCILDPP
ht-a            RQRPSYEFSDCSLNHYRTFIINYNPQCILNEP
macaeap1        SIP-ALKFSKCSQNQYHQYLKDYKPTCMLNIP
musms2          GSKFPRIFSRCSKIDLESFVTKPQTGCLTNVP
musmela         GFPFPMVFSSCSRKDLEASLEKGMGMCLFNLP
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B Disintegrin-like domain

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                                                                    456
VAP1            LKTDVVSPAVCGNYFVEVGEECDCGSPRTCRDPCCDATTCKLRQGAQCAEGLCCDQCRFK
agkis           NGTDIVSPPVCGNELLEVGEECDCGSPTNCQNPCCDAATCKLTPGSQCADGVCCDQCRFT
ecarin          LRKDIASPAVCGNEIWEEGEECDCGSPADCRNPCCDAATCKLKPGAECGNGECCDKCKIR
ht-a            LQTDIISPPVCGNELLEVGEECDCGSPRTCRDPCCDAATCKLHSWVECESGECCQQCKFT
macaeap1        FPCNFDDFQFCGNKKLDEGEECDCGPPQECTNPCCDAHTCVLKPGFTCAEGECCESCQIK
musms2          DVNRFVGGPVCGNLFVEHGEQCDCGTPQDCQNPCCNATTCQLVKGAECASGTCCHECKVK
musmela         EVKQAFGGRKCGNGYVEEGEECDCGEPEECTNRCCNATTCTLKPDAVCAHGQCCEDCQLK

                                                        488
VAP1            GAGTECRAAKDECDMADVCTGRSAEC-TDRFQR
agkis           RAGTECRQAKDDCDMADLCTGQSAECPTDRFQR
ecarin          KAGTECRPARDDCDVAEHCTGQSAECPRNEFQR
ht-a            SAGNVCRPARSECDIAESCTGQSADCPTDDFHR
macaeap1        KAGSICRPAEDECDFPEMCTGHSPACPKDQFRV
musms2          PAGEVCRLSKDKCDLEEFCDGRKPTCPEDAFQQ
musmela         PPGTACRGSSNSCDLPEFCTGTAPHCPANVYLH
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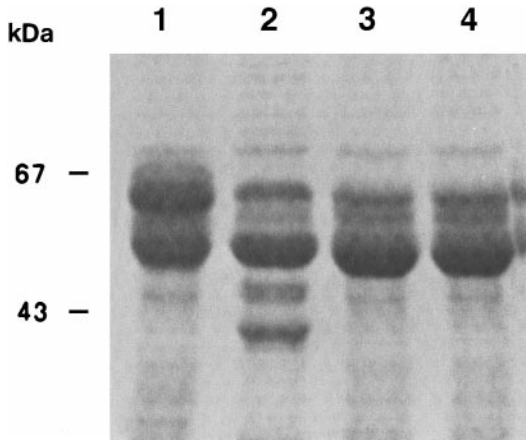

FIG. 3. Proteolysis of fibrinogen by VAP1. VAP1 was incubated in 10 mM sodium phosphate buffer (pH 7.0) with 0.01% SDS for 3 h at 37°C. The incubation was done in the presence or absence of either 4 mM EDTA or 4 mM EGTA. Fibrinogen (100 μg) was incubated with above-mentioned VAP1 (0.5 μg) for 24 h at 37°C. After incubation, samples were fractionated by SDS–PAGE (12% polyacrylamide) and stained with Coomassie brilliant blue. Lane 1, fibrinogen incubated alone; lane 2, fibrinogen incubated with VAP1; lane 3, fibrinogen incubated with VAP1 that had previously been incubated with 4 mM EDTA; lane 4, fibrinogen incubated with VAP1 that had previously been incubated with 4 mM EGTA.

from *C. atrox* (9, 10). In the present study, we cloned VAP1 cDNA using a cDNA library prepared from the venom glands of *C. atrox*. The deduced amino acid sequence of VAP1 consisted of 610 amino acids with a calculated molecular mass of 67 kDa. The calculated molecular mass of VAP1 was larger than the 55 kDa determined for the monomeric form by SDS–PAGE (9). Analysis of the cDNA sequence revealed that VAP1 is a multidomain molecule with a proprotein domain, a metalloprotease domain, a disintegrin-like domain, and a cysteine-rich domain. The multidomain structure is in agreement with the common precursor model of snake venom metalloprotease/disintegrin (21). If we assume that the proprotein domain is cleaved during posttranslational modification, the molecular mass of the mature VAP1 monomer would be 47 kDa. This value is closer to the molecular mass of 55 kDa determined by SDS–PAGE.

The deduced sequence of VAP1 included the consensus sequence of a zinc-binding site, H-E-X-X-H-X-X-G-X-X-H, in the metalloprotease domain. We noted previously that VAP1 had no detectable proteolytic activity against bovine serum albumin (9). However, VAP1 did degrade fibrinogen unless VAP1 had been incubated first in the presence of EDTA or EGTA. Inhibition of proteolytic activity by EDTA or EGTA is a typical characteristic of metalloproteases. By contrast, hydrolysis of fibrinogen by VAP1 was not inhibited by phenylmethylsulfonyl fluoride (PMSF; data not shown). Thus, the hydrolysis of fibrinogen appears to have been due to the metalloprotease activity of VAP1.

The proteolytic activity of VAP1 seemed to be necessary for the induction of apoptosis in VEC. When VEC were treated with VAP1, we observed the morphological and biochemical changes that are typical of apoptosis. However, when VEC were treated with VAP1 that had previously been incubated with EDTA or EGTA, none of these morphological and biochemical changes were observed. By contrast, treatment of VAP1 with PMSF had no effect on the induction of apoptosis by VAP1 in VEC (data not shown). These results suggest

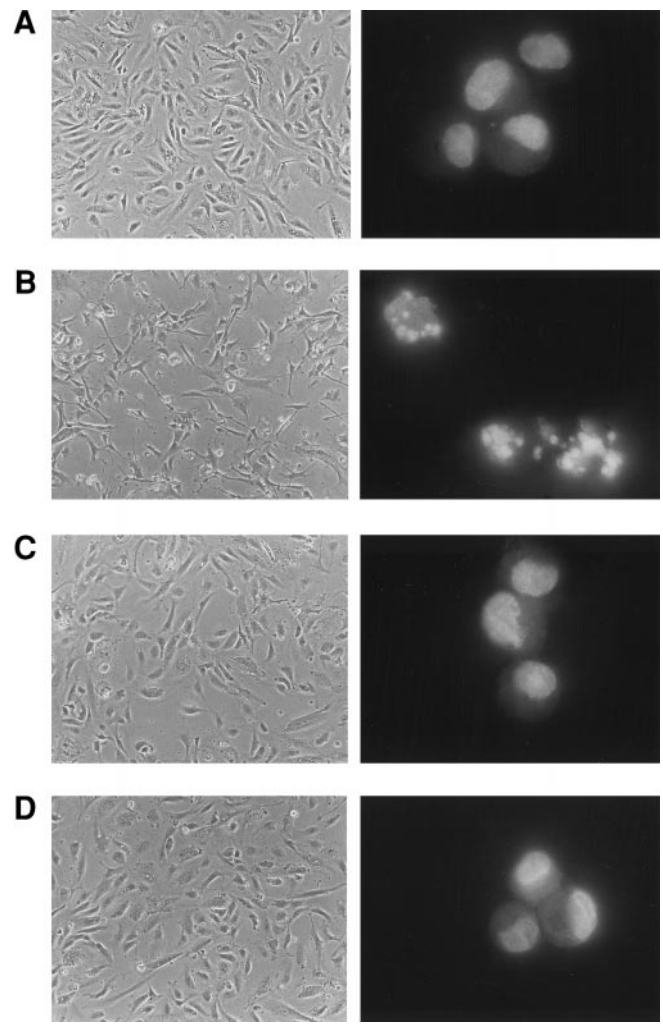

FIG. 4. Induction of apoptosis by VAP1. VAP1 was incubated in 10 mM sodium phosphate buffer (pH 7.0) with 0.01% SDS for 3 h at 37°C. The incubation was done in the presence or absence of either 2 mM EDTA or 2 mM EGTA. VEC were incubated with or without above-mentioned VAP1. A, VEC; B, VEC that had been incubated with VAP1 (1 μg/ml); C, VEC that had been incubated with VAP1 after incubation of VAP1 with 2 mM EDTA; D, VEC that had been incubated with VAP1 after incubation of VAP1 with 2 mM EGTA. Left: After treatment for 18 h, cells were photographed under a light microscope. Magnification, ×100. Right: After treatment for 12 h, cells were fixed with glutaraldehyde and stained with Hoechst 33258. Nuclear fragmentation was examined by fluorescence microscopy. Magnification, ×400.

that the metalloprotease activity of VAP1 might be involved in induction of apoptosis in VEC.

Sequence analysis revealed that VAP1 is similar to members of the metalloprotease/disintegrin family. In the induction of hemorrhage, the members of the metalloprotease/disintegrin family in hemorrhagic snake venoms each function effectively against their own respective targets; platelets are targets for disintegrins; basement membranes are targets for hemorrhagic proteinases; and fibrinogen is the target for fibrinogenases (11, 12, 22). In concert with these proteins, VAP1 might act on endothelial cells to induce hemorrhage. The amino acid sequence of VAP1 is also similar to that of the members of the ADAM family. The ADAMs are a recently discovered family of membrane-anchored glycoproteins comprising a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain and a cytoplasmic domain. Almost 30 family members have been identified, some of these have been proposed as candidates for modulating proteolysis, cell adhesion, cell fusion, and signaling. However, the functions of most other family members are not clear. Although all ADAMs have a relatively well-conserved metalloprotease domain, only half of those identified to date contain the zinc-binding consensus sequence. Thus, only half of the known ADAMs are predicted to be catalytically active, whereas the others most likely lack metalloprotease activity. Indeed, protease activity have been demonstrated so far for only a few members of the ADAMs, for example, TACE and KUZ. TACE and KUZ have been suggested to be involved in protein ectodomain shedding (23, 24), an important regulatory step in the function of membrane proteins involved in cell-cell communication during development, cell differentiation and tissue maintenance. Similarly, VAP1 might act on VEC to cleave a membrane-anchored protein, as a specific substrate, to induce apoptosis.

Disintegrins include a highly conserved RGD sequence and they competitively block the adhesive functions of RGD-dependent integrins in various cell types (13). In other metalloprotease/disintegrins in snake venoms, this sequence is replaced by sequences, such as SECD and DDCD (19, 25–27). In its disintegrin-like domain, VAP1 has DECD in the positions that correspond to those of RGD. The DECD sequence has also been found in fertilin β, which belong to the ADAM family. Fertilin acts as the sperm ligand that interacts with integrin $\alpha 6\beta 1$ on the plasma membrane of the egg (28). VAP1 might, therefore, interact with a specific surface protein to induce apoptosis in VEC.

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