A New Gene Structure of the Disintegrin Family: A Subunit of Dimeric Disintegrin Has a Short Coding Region†

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ABSTRACT: Disintegrin is a potent platelet aggregation inhibitor isolated from various snake venoms. The cDNA of the snake venom disintegrin family precursor is well-known to encode pre-peptide, metalloprotease, spacer, and disintegrin domains. Recently, new types of disintegrins, dimeric disintegrins, have been isolated, and their amino acid sequences were determined to be ∼65 amino acid residues in each subunit. We isolated a novel heterodimeric disintegrin, acostatin, from the venom of *Agkistrodon contortrix contortrix*, which consisted of 63 and 64 amino acid residues in the α chain and β chain, and both chains had the Arg−Gly−Asp (RGD) sequence for binding platelet GPIIb/IIIa. The cDNA lengths of the α chain and the β chain of acostatin were 902 bp and 2031 bp, respectively. The acostatin α chain precursor, surprisingly, has the only disintegrin domain alone and lacked almost all of the pre-peptide and metalloprotease domains. The precursor of the acostatin β chain belongs to a well-known motif of disintegrin precursors. Furthermore, both precursors of α and β chains of another heterodimeric disintegrin, piscivostatin, also have the same domain structures as those of acostatin subunits. These results indicate that the cDNAs of heterodimeric disintegrin subunits have quite a different length of coding region and their precursors have a novel domain structure of disintegrin-family proteins.

Disintegrins with ∼80 amino acid residues are potent platelet aggregation inhibitors derived from snake venom. They have an Arg−Gly−Asp (RGD)[1] or a Lys−Gly−Asp (KGD) sequence in their amino acid sequence for the binding motif to several integrins, such as platelet GPIIb/IIIa complex (*1*−*3*). Over the past years, around 40 disintegrins have been isolated and characterized from many snake venoms. However, the cDNA cloning of only a few disintegrins has been reported so far (*4*−*10*). Interestingly, venom disintegrins are encoded with a pre-peptide, metalloprotease, and disintegrin region on their common precursor. It is suggested that the metalloprotease/disintegrin precursor is cleaved by protease(s), resulting in production of metalloprotease and disintegrin (*11*). Many venom metalloproteases also contain a disintegrin-like domain with no RGD or KGD sequence and a cysteine(Cys)-rich domain in those structures (*12*−*14*). The metalloprotease/disintegrin(/Cys-rich) domain structures are commonly distributed in reptiles and mammals. This disintegrin-like domain is found in the mammalian ADAM (A disintegrin and metalloprotease) family proteins such as MDC9 (*15*), fertilin-α (*16*), and TACE (TNF-α converting enzyme) (*17*) and ADAM-TS (thrombospondin) family proteins such as aggrecanase-1 (*18*) and -2 (*19*), indicating that the disintegrin-like domain is involved in cell−matrix interaction (*20*, *21*).

In this study, we show the cDNA cloning of two heterodimeric disintegrins, acostatin and piscivostatin, from the venom of two different snake species. Both subunits of acostatin and piscivostatin have apparently different-length mature protein-coding regions from the other members of the venom metalloprotease/disintegrin family. One of two subunits in acostatin and piscivostatin has a shorter length cDNA than other members of venom metalloprotease/disintegrin family. This is the first report of the cDNA cloning of heterodimeric disintegrin and a novel domain structure of the disintegrin family gene.

MATERIALS AND METHODS

Materials. The lyophilized venom of *Agkistrodon contortrix contortrix* and *Agkistrodon piscivorus piscivorus* was purchased from the Kentucky Reptile Zoo. Superdex 75 pg and SP-Sepharose High Performance FPLC columns were from Amersham Pharmacia LKB Biotechnology Inc. COSMOSIL 5C18 AR-300 HPLC column was purchased from Nacalai Tesque (Kyoto, Japan). Endoprotease Lys-C was purchased from Seikagaku Corporation (Tokyo, Japan). Endoprotease Asp-N was purchased from Boehringer Mannheim (Marburg, Germany). ADP was purchased from Sigma (MO). Perfect RNA Markers purchased from Novagen. (WI). Chemicals used in this study were purchased from Amersham Pharmacia Biotech, Sigma, Wako Pure Chemical Corporation (Osaka, Japan), and Kanto Chemical Corporation (Tokyo, Japan).

Purification of Acostatin. One gram of lyophilized venom was dissolved in 50 mM Tris−HCl buffer (pH 8.0), and the insoluble materials were removed by centrifugation. The

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[1] Abbreviations: RGD, arginine−glycine−aspartate; KGD, lysine−glycine−aspartate; GPIIb/IIIa, glycoprotein IIb/IIIa; PRP, platelet-rich plasma; PPP, platelet-poor plasma; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ADAM, A disintegrin and metalloprotease.

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supernatant was applied to a column of Superdex 75 pg (1.6 × 60 cm), preequilibrated with the same buffer, and eluted at a flow rate of 1 mL/min. Fractions were assayed for inhibitory activity on ADP-induced aggregation of human platelets. The active fractions were pooled and then loaded onto a column of SP-Sepharose High Performance (1.6 × 10 cm) preequilibrated with 20 mM imidazole−HCl buffer (pH 6.8). The column was washed with the same buffer and developed with a linear gradient of NaCl (0−0.4 M) in the same buffer over 80 min at a flow rate of 2 mL/min. The active fractions were pooled and loaded onto a preparative COSMOSIL 5C18 reverse-phase HPLC column (0.45 × 25 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid in water. The column was developed at room temperature with a linear acetonitrile gradient in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 mL/min.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The apparent M_r of acostatin was estimated using a Tris−tricine 16% gel with molecular weight markers (Pharmacia Biotech) according to the protocol of Schägger and von Jagow under reducing and nonreducing conditions (*22*).

Amino Acid Sequence Analysis. Acostatin was reduced for 1 h at room temperature with 20 mM dithiothreitol in the presence of 0.5 M Tris−HCl, 6 M guanidinium hydrochloride and 2 mM EDTA (pH 8.3) in a volume of 0.5 mL. 4-Vinylpyridine was then added, and alkylation was allowed to proceed for 1 h at room temperature. The *S*-pyridylethylated α and β chains of acostatin were separated from the reagents by C18 reverse-phase HPLC, and their complete amino acid sequences were determined by sequencing the peptides obtained by digestion with endoprotease Asp-N and endoprotease Lys-C. All samples were analyzed on Applied Biosystems protein sequencers (model 473A and model 477A).

Platelet Aggregation Studies. Total human blood was obtained from healthy volunteers with informed consent; all the volunteers denied taking any medications. The study protocol is approved by the Ethics Committee of our university. Blood was collected from volunteers into a 0.1 volume of 3.8 w/v% sodium citrate by venipuncture and then centrifuged at 150*g* for 20 min at room temperature to prepare platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was separated from the PRP by further centrifugation at 300*g* for 5 min. Disintegrin was added to the platelets and incubated at 37 C°, followed by addition of ADP (10 μM final concentration) to initiate aggregation. Platelet aggregation was measured by determining the changes in light transmission with an optical aggregometer (Niko Bioscience Hema Tracer 601) and AG-10 (Kowa, Tokyo, Japan).

cDNA Cloning of Acostatin and Piscivostatins.[2] cDNA cloning of disintegrins was carried out by a RT-PCR method. The total RNA was isolated from the venom gland of *Agkistrodon contortrix contortrix* and *Agkistrodon piscivorus piscivorus* with ISOGEN (Wako Pure Chemical Co., Osaka, Japan) according to the manufacturer's protocol. The templates of RACE-PCR were prepared by RT-PCR using the MMLV reverse transcriptase (GIBCO, MD). 5′- and 3′-RACE were carried out to determine the nucleotide sequence of 5′- and 3′-end cDNA using the SMART RACE cDNA amplification kit (CLONTECH, CA), with degenerated primers based on partial amino acid sequences and single primers identified as nucleotide sequences (5′-at(a/t/c) aa-(a/g) gct gg(a/t/g/c) aa(a/g) at(a/t/c) tg-3′ for acostatin α chain of 3-RACE, annealing condition was 49 °C; 5′-ga(t/c) gct cc(a/t/g/c) gct aa(t/c) cc(a/t/g/c) tg-3′ for acostatin and piscivostatin β chain of 3-RACE, annealing condition was 46 °C; 5′-at(a/t/c) ca(a/g) cc(a/t/g/c) aa(a/g) aa(t/c) cc(a/t/g/c) tg-3′ for piscivostatin α chain 3-RACE, annealing condition was 46 °C; 5′-ggc aga tgg gtc tct ttg gcc tac-3′ for acostatin and piscivostatin α chain of 5-RACE, annealing condition was 57 °C; 5′-agg cag atg gat ctc ttt gga ttt g-3′ for 5-RACE of acostatin β chain, annealing condition was 57 °C; 5′-tgg gac agc cag cag ata tgc cat tgc agt a-3′ for piscivostatin β chain 5-RACE, annealing condition was 57 °C). The RACE products were subcloned into pGEM T-easy vector (Promega, WI) and transformed to *E. coli* XL-1 blue. The positive clones identified by blue-white color selection system and sequenced using DNA sequencer DSQ 2000L (Shimadzu, Kyoto, Japan).

Northern Blot Analysis. Alkaline phosphatase-labeled partial cDNA of acostatin α chain and β chain cDNA were used as a probe for analysis of acostatin and piscivostatin mRNA length. We prepared three DNA probes: A, 5′-UTR to disintegrin region of acostatin α chain (nucleotide position −80 to 361 of acostatin α chain in Figure 3); B, disintegrin region to 3′-UTR of acostatin α chain (nucleotide position 151−783 of α chain in Figure 3); and C, metalloprotease region of acostatin β chain (nucleotide position 494−1032 of β chain in Figure 4). mRNA was isolated from the venom gland total RNA using Oligotex-dT30⟨*super*⟩ mRNA purification kit (TaKaRa, Shiga, Japan). The protocol of Northern blotting was in accordance with the description in Molecular Cloning 3rd Ed (*23*) and the manufacturer's protocol of Alkphos Direct (Amersham Pharmacia Biotech, U.K.). In summary, mRNA was electrophoresed in denatured agarose gel and transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech). Hybridization with DNA probes was performed under stringent conditions at 55 °C for 15 h, and then the membrane was washed and the signal was detected with detection reagent (CDP-*star* chemiluminescent detection reagent, Amersham Pharmacia Biotech, UK) by exposing to X-ray film for 30 min.

RESULTS

Purification of Acostatin of Dimeric Disintegrin. The presence of disintegrin in the venom of *Agkistrodon contortrix contortrix* was examined by platelet aggregation inhibitory assay on both platelet-rich plasma (PRP) and washed platelets. Finally, two platelet aggregation inhibitors, which designated acostatin 1 and acostatin 2, were isolated by preparative reversed-phase C18 HPLC column. Acostatin 1 and 2 was eluted at 22% and 25% acetonitrile, respectively (Figure 1A). The yields of acostatin 1 and acostatin 2 were 3.8 and 5.2 mg from 1 g of venom, respectively. The apparent M_r on SDS-PAGE of acostatin 1 and acostatin 2 was 16 kDa under nonreducing conditions and 8 kDa under reducing conditions on SDS-PAGE (inset in Figure 1A).

Characterization of Acostatin. The *S*-pyridylethylated acostatin 2 was separated to two peaks by C18 HPLC (Figure

[2] Genbank accession numbers: acostatin α chain, AB078903; acostatin β chain, AB078904; piscivostatin α chain, AB078905; piscivostatin β chain, AB078906; HR2a/flavostatin precursor, AY037808.

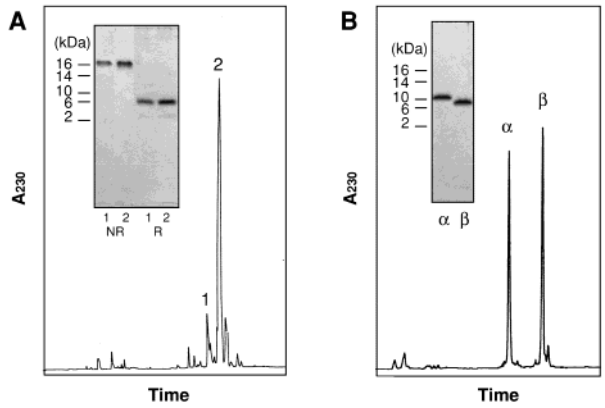

FIGURE 1: Purification and *S*-pyridylethylation of acostatin. (A) HPLC purification of acostatin. The numbers 1 and 2 on the peaks indicate acostatin 1 and acostatin 2, respectively. The inset shows the SDS-PAGE of acostatin 1 and 2 under nonreducing (NR) and reducing (R) conditions. (B) HPLC purification of *S*-pyridylethylated subunits of acostatin 2. The symbols of α and *β* on each peak show the α chain and *β* chain of acostatin 2, respectively. The inset shows the SDS-PAGE of Pe-α chain and Pe-*β* chain of acostatin under nonreducing condition.

1B): α chain and *β* chain were eluted at 26%, 29%, respectively. The apparent M_r of *S*-pyridylethylated α chain and *β* chain were determined to be 10.5 and 9.6 kDa by SDS-PAGE (inset in Figure 1B). The chromatographic pattern of acostatin 1 was essentially identical to that of acostatin 2 (data not shown). The amino acid sequences of acostatin 1 and acostatin 2 were determined by Edman degradation of each *S*-pyridylethylated sample, endoprotease Lys-C and endoprotease Asp-N digested fragments. The complete amino acid sequences of acostatin 2 are shown in Figure 2A. Acostatin 2 was composed of an α chain of 63 amino acids and a *β* chain of 64 amino acids (Figure 2A). Both the α chain and *β* chains contain the Arg−Gly−Asp (RGD) sequence for binding to RGD-dependent integrins such as GPIIb/IIIa. At the determination of amino acid sequence, the yield of the α chain in the sequencing reaction was much less than that of the *β* chain (about 20%). The peptide fragment D4 in the α chain did not detect any PTH-amino acids. The amino acid composition of fragment D4 [Asp(1), Cys(2), Glu(1), Lys(1), Pro(2)] accords with the composition of the N-terminal region, ^{2}Gln−^{8}Cys, of the α chain (see Figure 2, deduced amino acid sequence in parentheses). The masses of acostatin 2, 13 510.2 and 13 641.3, were detected when the preparation of acostatin 2 was measured by MALDI-TOF mass spectrometry. The

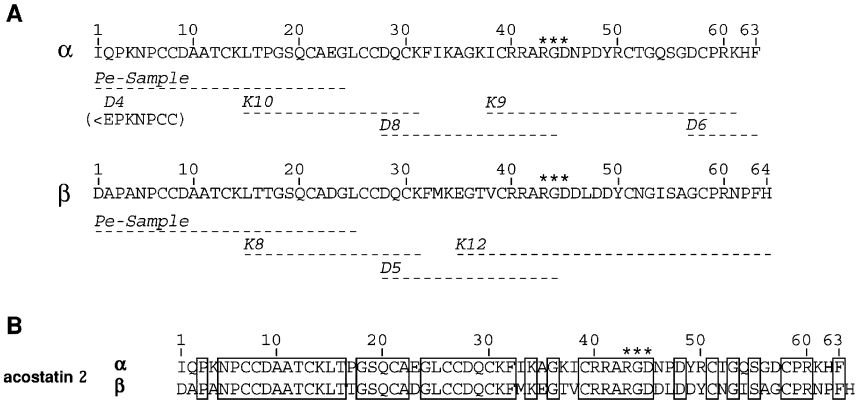

FIGURE 2: Amino acid sequence of acostatin 2. (A) Amino acid sequence of acostatin 2. Amino acids are shown by a one-letter code. Asterisks show the position of the RGD sequence in each subunit. Pe-sample, *S*-pyridylethylated sample; K, Lys-C fragment; D, Asp-N fragment. (B) Comparison of amino acid sequence of acostatin subunits. Boxes show the identical amino acids, and the positions of the RGD sequence are indicated by asterisks.

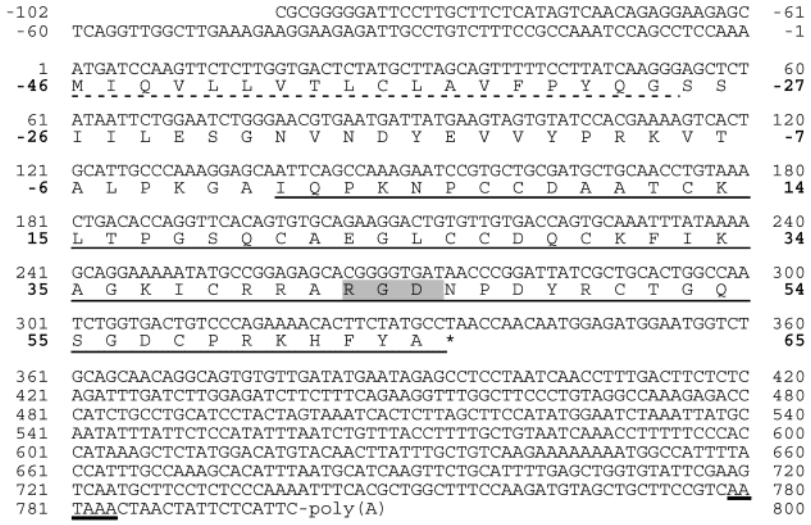

FIGURE 3: cDNA and deduced amino acid sequence of acostatin α chain precursor. Nucleotide and amino acid (bold) number are indicated on both sides of the sequence. Number 1 indicates the position of start codon and the N-terminus of acostatin α chain, respectively. The putative signal peptide, mature acostatin α chain, RGD sequence, and polyadenylation signal are indicated by dotted underline, single underline, shadow, and bold underline, respectively.

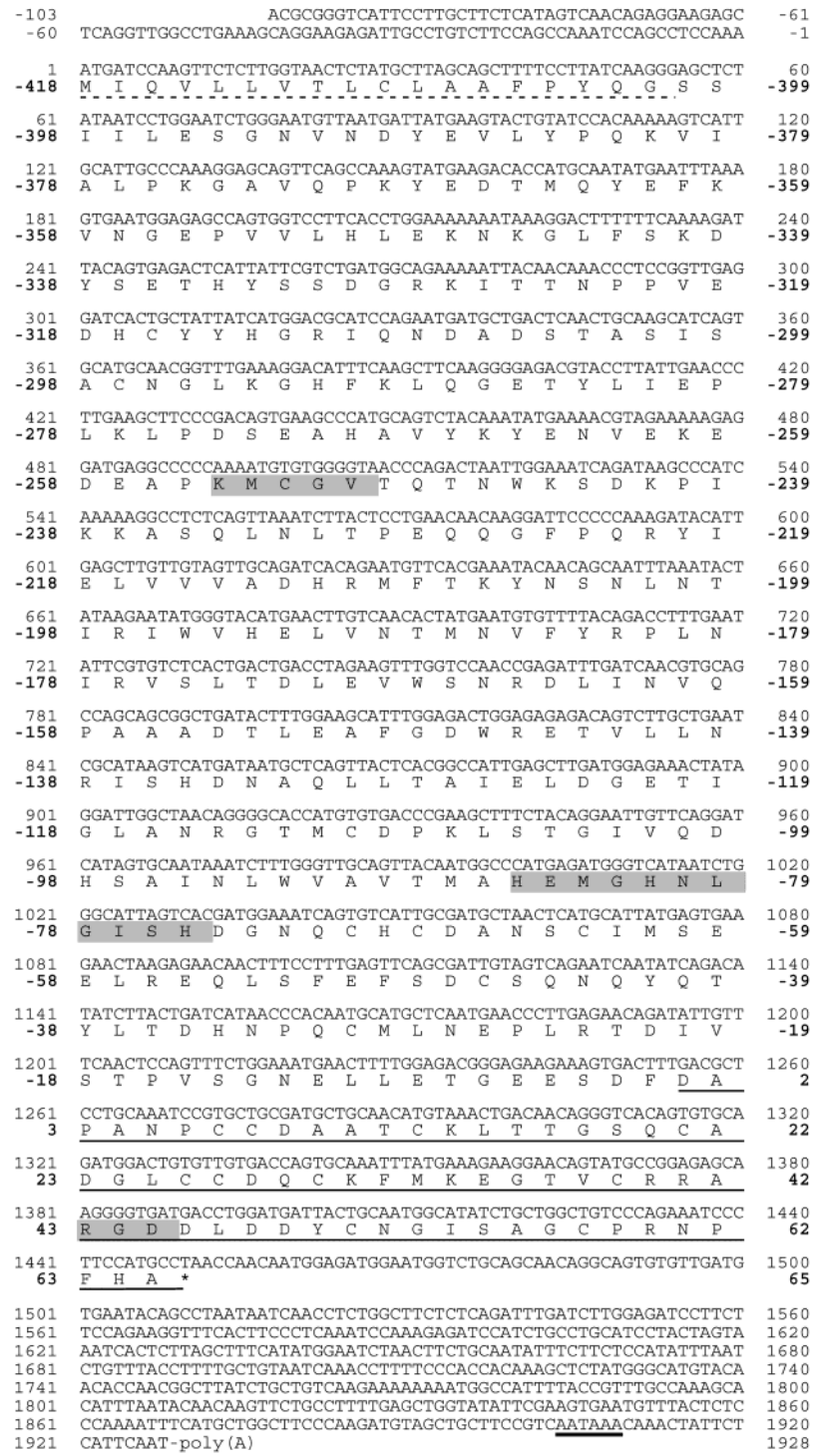

FIGURE 4: cDNA and deduced amino acid sequence of acostatin β chain precursor. The number of nucleotide and amino acid (bold) are indicated on both sides of the sequence. Number 1 indicates both position of start codon and N-terminus of acostatin β chain, respectively. The putative signal peptide, mature acostatin β chain, and polyadenylation signal are indicated by dotted underline, single underline, and bold underline, respectively. The positions of Cys-switch site (KMCGV), Zn^{2+}-binding motif (HEMGHNLGISH), and RGD sequence are shaded.

difference between both masses, 130.1, is consistent with the mass of pyroglutamic acid, the N-terminus of the α chain, indicating that the preparation acostatin 2 contained the mixture of ^{1}Ile-lacking form and intact acostatin.

The amino acid sequence of acostatin 1 was identical to that of acostatin 2, except for lacking the two C-terminal amino acid residues (^{62}His and ^{63}Phe) in the α chain of acostatin 2, and those of the β chains of acostatin 1 and acostatin 2 were identical (data not shown). Acostatin 1 is most likely to be a degradation form of acostatin 2 by some protease(s) because we did not clone the acostatin 1 α chain, which end the open reading frame at the site of ^{61}Lys.

Platelet Aggregation Inhibitory Activity of Acostatin. Both acostatins inhibited ADP-induced platelet aggregation in human PRP in a dose-dependent manner. The IC50 value for 10 μM ADP-induced platelet aggregation of acostatin 1 and acostatin2 were 65 ± 19 and 72 ± 30 nM (n = 3), respectively.

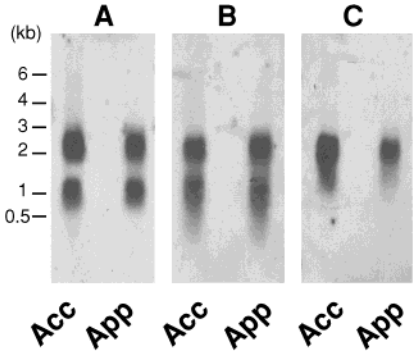

FIGURE 5: Northern blot analysis of acostatin and piscivostatin mRNAs. Acostatin and piscivostatin mRNAs were detected by DNA probes, which involves nucleotide sequence of 5′-UTR to disintegrin region in acostatin α chain (A), disintegrin region to 3′-UTR in acostatin α chain (B), and metalloprotease region of acostatin β chain (C), respectively. Acc, *Agkistrodon contortrix contortrix*; App, *Agkistrodon piscivorus piscivorus*.

We have recently reported the characterization of a dimeric disintegrin, piscivostatin, from the venom of *Agkistrodon piscivorus piscivorus* (*24*). Piscivostatin has a unique effect on platelet aggregation, which inhibits both platelet aggregation and platelet aggregate dissociation (*24*). Acostatin has essentially identical biological activity to that of piscivostatin on platelet aggregation (data not shown).

cDNA Cloning of Acostatin and Piscivostatin. The cDNAs encoding acostatin subunit precursors were obtained by PCR. The nucleotide sequence of the acostatin α chain precursor of 902 bp contained a 5′- untranslated region (5′-UTR) of 102 bp, an open reading frame (ORF) of 333 bp, including a signal peptide/pre-peptide domain of 138 bp and an acostatin α chain of 195 bp, a stop codon, and a 3′-untranslated region (3′-UTR) of 464 bp (Figure 3). The acostatin β chain precursor of 2031 bp had a 5′-UTR of 103 bp, an ORF of 1449 bp, including a signal peptide/pre-peptide/metalloprotease domain of 1254 bp and an acostatin β chain of 195 bp, a stop codon, and 3′-UTR of 476 bp (Figure 4).

The nucleotide sequence of the piscivostatin α chain precursor had 937 bp, which is 94.8% identical to the acostatin α chain precursor, and that of the β chain precursor had 2029 bp, which is 98.2% identical to the acostatin β chain precursor (data not shown). The deduced amino acid sequences in the ORF of piscivostatin subunits are shown in Figure 6.

Northern Blot Analyses of Heterodimeric Disintegrin mRNAs. Northern blots were performed using three DNA probes amplified from acostatin α chain and β chain cDNA, which has 5′-UTR to the disintegrin region of α chain precursor (probe A), disintegrin region to 3′-UTR of α chain precursor (probe B), and the metalloprotease region of β chain precursor (probe C). The mRNA lengths of the acostatin and piscivostatin subunits were estimated by R_f values of RNA electrophoresis standard marker. Both α chain DNA probes detected two signals indicating mRNA lengths of approximately 2.2 and 1.1 kb (Figure 5A,B), while β chain DNA probe detect only 2.2 kb signal (Figure 5C), respectively.

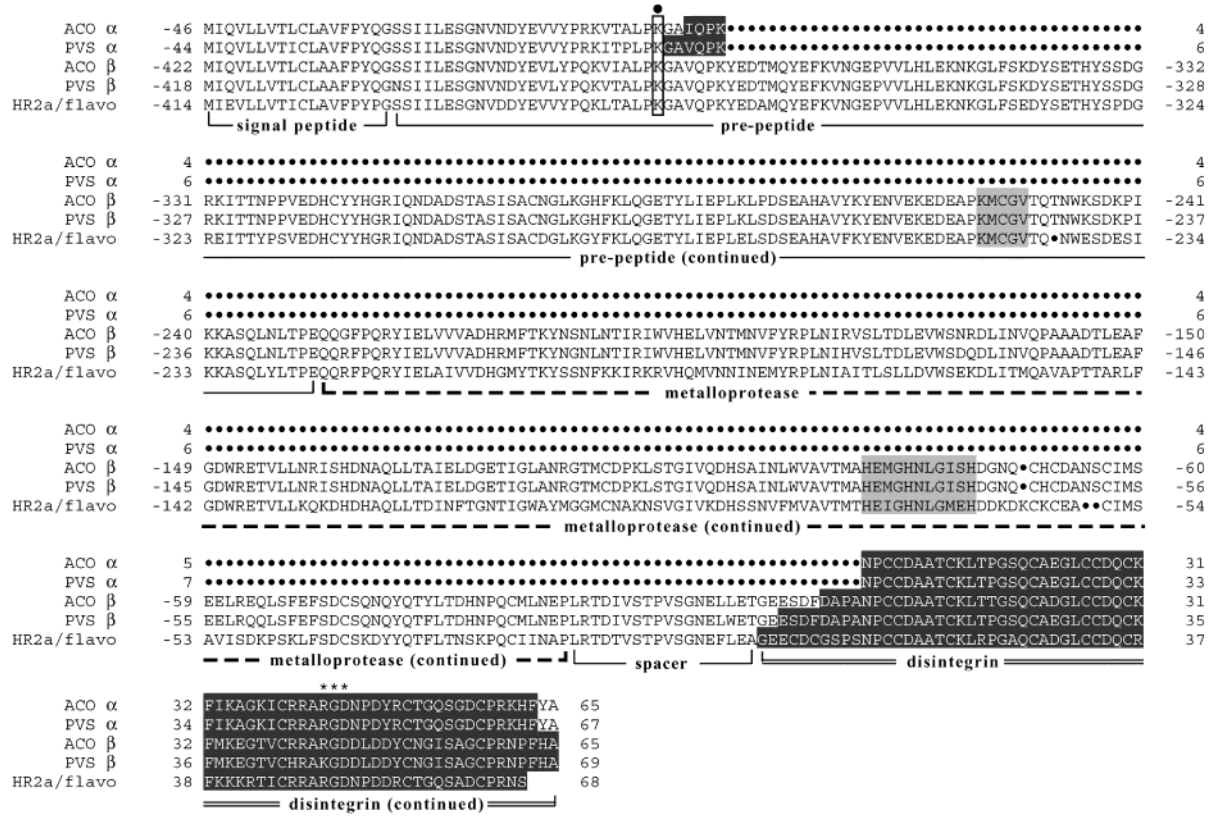

FIGURE 6: Comparison of deduced amino acid sequence in open reading frame regions of precursor for acostatin and piscivostatin subunits and HR2a/flavostatin. Shaded amino acids (cysteine-switch site and Zn^{2+}-binding motif) and asterisks (RGD/KGD sequence). Reversed characters show the mature disintegrins. The boxed Lys residues with closed circle, for example, $^{-3}$Lys in acostatin α chain, show the putative cleavage site of the disintegrin precursors. Number 1 indicates the N-terminus of disintegrin. ACO α, acostatin α chain; ACO β, acostatin β chain, PVS α, piscivostatin α chain; PVS β, piscivostatin β chain; HR2a/flavo, HR2a/flavostatin (*9*). Genbank accession numbers: acostatin α chain, AB078903; acostatin β chain, AB078904; piscivostatin α chain, AB078905; piscivostatin β chain, AB078906; HR2a/flavostatin precursor, AY037808.

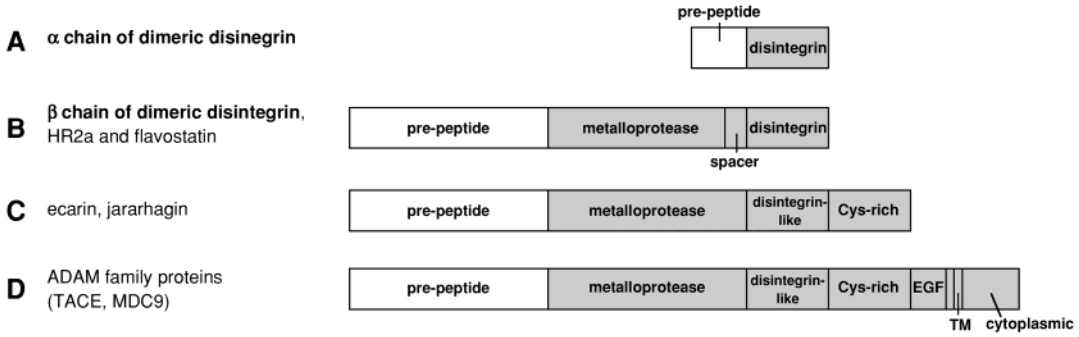

FIGURE 7: Schematic domain structure models of snake venom and mammalian metalloprotease/disintegrin family. Shaded regions indicate the mature protein region. EGF, EGF (epidermal growth factor)-like domain; TM, transmembrane domain.

DISCUSSION

In this study, we isolated acostatin from the venom of *Agkistrodon contortrix contortrix* and performed cDNA cloning of both acostatin and piscivostatin, which previously isolated from the venom of *Agkistrodon piscivorus piscivorus* (*24*). The cDNA sequences of both dimeric disintegrins encoding subunits revealed quite different lengths of open reading frame to each other. It was found that both α chain precursors of acostatin and piscivostatin have a novel domain structure in all members of the snake metalloprotease/disintegrin family and mammalian ADAM (A disintegrin and metalloprotease) family proteins.

As shown in Figures 3 and 4, the nucleotide length of acostatin α chain (902 bp) has only half of that of *β* chain (2031 bp). The piscivostatin subunits have similar length of cDNAs against acostatin. The cDNA length of piscivostatin α chain was 937 bp, while that of *β* chain was 2029 bp. It has been reported that the snake venom disintegrin is encoded with metalloprotease in all cDNAs of disintegrins so far. Both hemorrhagic metalloprotease HR2a and monomeric disintegrin flavostatin are encoded on a single precursor (*9*), and the cDNAs of many snake venom metalloproteases and mammalian ADAM family proteins have a disintegrin-like domain in their nucleotide sequence (*7*, *12*−*17*, *31*, *32*). To confirm the mRNA length of α chains of acostatin and piscivostatin, we prepared DNA probes from cDNA sequences in the acostatin α chain and *β* chain and examined by Northern blot analysis against each mRNA, which was purified from the venom glands total RNA of *A. contortrix contortrix* and *A. piscivorus piscivorus*. The DNA probes derived from α chain detected two mRNA signals of 2.2 and 1.1 kb (Figure 5A,B), and the DNA probe derived from *β* chain metalloprotease region detected only 2.2 kb signal (Figure 5C). The 1.1 kb signals indicate the mRNA of the acostatin and piscivostatin α chain precursor because the length of 1.1 kb is in accord with the cDNA length of the acostatin α chain of 902 bp and piscivostatin α chain of 937 bp plus hundreds of poly(A) tails. The identities among the acostatin α chain cDNA to acostatin, piscivostatin *β* chains, and other members of the metalloprotease/disintegrin family are over 80%. Therefore, the DNA probes of the acostatin α chain reacted not only with the mRNA of acostatin and piscivostatin α chain but also with *β* chain and many other homologous snake venom metalloprotease/disintegrin precursors. These results provide the strong evidence that both α chain precursors of the acostatin and piscivostatin were encoded on the shortest mRNA in venom metalloprotease/disintegrin and ADAM family proteins.

The comparison of deduced amino acid sequence in the open reading frame of acostatin, piscivostatin subunits and HR2a/flavostatin precursor (*9*) is shown in Figure 6. The α chain precursors of acostatin and piscivostatin consist of signal peptide, short pre-peptide domain (about 30 amino acids), and disintegrin domain. Both *β* chain precursors of acostatin and piscivostatin and HR2a/flavostatin precursors have the same domain structure with conserved Cys-switch site, which represses the protease activity, and Zn^{2+}-binding motif.

Interestingly, the amino acid sequences of the N-terminal portions of the acostatin α chain (GAIQPK sequence, $^{-2}$Gly to ^{4}Lys) and piscivostatin α chain (GAVQPK sequence, ^{1}Gly to ^{6}Lys) have an identity against the pre-peptide domain of acostatin, piscivostatin *β* chains, and flavostatin precursors, as shown in Figure 6. These results strongly suggest that these N-terminal regions of α chains precursor should be derived from the pre-peptide domain of snake venom disintegrin precursor. $^{-2}$Gly−$^{-1}$Ala in acostatin α chain precursor are most likely to digest by unknown protease such as an aminopeptidase during the process of maturation or after the maturation,

In Figure 6, boxed $^{-3}$Lys residues of acostatin α chain are a highly conserved amino acid residue in precursors of snake venom metalloprotease/disintegrin family proteins. We predict that the conserved Lys residue in pre-peptide region should be one of the cleavage sites by protease(s) in the maturation process of dimeric disintegrin precursor.

Both acostatin subunits and other dimeric disintegrins such as lebein (*27*), EC3 (*28*), and EMF10 (*29*) have a high degree of similarity each other (Figure 2B). The *β* chain of acostatin is completely identical to the deduced amino acid sequence of contortrostatin, which was reported as a homodimeric disintegrin from same venom and cloned its nucleotide sequence (*25*, *26*). We could not isolate any homodimeric disintegrin such as α/α-type and *β*/*β*-type from the three each different lots of venom of *Agkistrodon contortrix contortrix* and *Agkistrodon piscivorus piscivorus*.

Acostatin subunits have totally identical alignment of cysteine residues to EMF10. This suggests that acostatin has the same disulfide bridge patterns to EMF10 (*30*). Calvate et al. recently reported the disulfide bridge pattern of a heterodimeric disintegrin, EMF10. Two intrachain disulfide bonds joined the subunits of EMF10 (*30*). Since the alignments of all cysteine residues of acostatin and piscivostatin are identical to that of EMF10, we consider that these have the same localization of two intrachain disulfide bridges against EMF10.

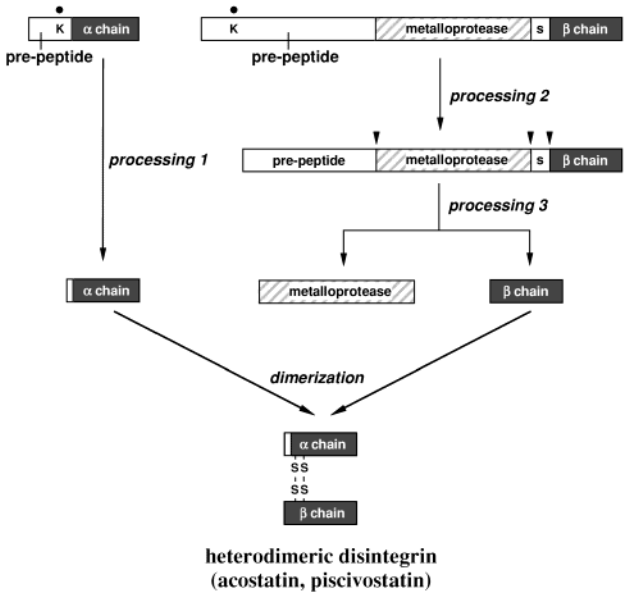

FIGURE 8: Schematic models of the maturation process of the precursor to matured proteins acostatin and piscivostatin. Arrowheads indicate the cleavage sites on disintegrin precursors by protease(s). The Lys residue with closed circle shows the potential cleavage site in pre-peptide during maturation of disintegrin precursor. Both subunits of acostatin and piscivostatin are bridged by two intrachain disulfide bonds (see Discussion).

The hypothesis of processing and dimerization steps of heterodimeric disintegrin is shown in Figure 8. The subunits of heterodimeric disintegrin cDNAs are coded on independent mRNAs. First, the cleavage occurs at the Lys residue (shown by K with closed circle) of both the α and β chain precursors (processing 1 and 2), and the α chain continues to finish maturation. Second, the β chain precursor is further digested to process the metalloprotease and mature β chain (processing 3). Finally, the mature β chain joins to the mature α chain by the formation of intrachain disulfide bridges to form α/β heterodimer.

In summary, we isolated and cloned two heterodimeric disintegrins, acostatin and piscivostatin, from the venom of two *Agkistrodon* species. Each subunit of both acostatin and piscivostatin is encoded on two different length cDNAs. The β chain precursors of acostatin and piscivostatin have a similar domain structure to the metalloprotease/disintegrin family. In contrast, the precursors of the α chain have a novel smaller domain structure, which consists of only pre-peptide, disintegrin domain, and no metalloprotease domain. This is the first report of cDNA cloning of heterodimeric disintegrin and of a novel domain structure in all disintegrin domain-containing genes.

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