Complete Amino Acid Sequence of Kaouthiagin, a Novel Cobra Venom Metalloproteinase with Two Disintegrin-like Sequences[†]

Masayuki Ito,[‡] Jiharu Hamako,[§] Yoshihiko Sakurai,[‖] Masanori Matsumoto,[‖] Yoshihiro Fujimura,[‖] Masami Suzuki,[‡] Keiichiro Hashimoto,[‡] Koiti Titani,[‡] and Taei Matsui*,[‡]

Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan, Department of Medical Information Technology, Fujita Health University College, Toyoake, Aichi 470-1192, Japan, and Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Nara 634-8522, Japan

ABSTRACT: The primary structure of kaouthiagin, a metalloproteinase from the venom of the cobra snake *Naja kaouthia* which specifically cleaves human von Willebrand factor (VWF), was determined by amino acid sequencing. Kaouthiagin is composed of 401 amino acid residues and one Asn-linked sugar chain. The sequence is highly similar to those of high-molecular mass snake venom metalloproteinases from viperid and crotalid venoms comprised of metalloproteinase, disintegrin-like, and Cys-rich domains. The metalloproteinase domain had a zinc-binding motif (HEXXHXXGXXH), which is highly conserved in the metzincin family. Kaouthiagin had an HDCD sequence in the disintegrin-like domain and uniquely had an RGD sequence in the Cys-rich domain. Metalloproteinase-inactivated kaouthiagin had no effect on VWF-induced platelet aggregation but still had an inhibitory effect on the collagen-induced platelet aggregation with an IC$_{50}$ of 0.2 μM, suggesting the presence of disintegrin-like activity in kaouthiagin. To examine the effects of these HDCD and RGD sequences, we prepared synthetic peptides cyclized by an S–S linkage. Both the synthetic cyclized peptides (RAAKHDCDLPELC from the disintegrin-like domain and CFDLNMRGDDGSFC from the Cys-rich domain) had an inhibitory effect on collagen-induced platelet aggregation with IC$_{50}$ values of ~90 and ~4.5 μM, respectively. The linear peptide (RAAKHDCDLPELC) and the cyclized peptide (CFDLNMRGEDGSFC) had little effect on collagen-induced platelet aggregation. These results suggest that kaouthiagin not only inhibits VWF-induced platelet aggregation by cleaving VWF but also disturbs the agonist-induced platelet aggregation by both the disintegrin-like domain and the RGD sequence in the Cys-rich domain. Furthermore, our results imply that the corresponding part of the Cys-rich domain in other snake venom metalloproteinases also has a synergistic disturbing effect on platelet aggregation, serving as a second disintegrin-like domain. This is the first report of an elapid venom metalloproteinase with two disintegrin-like sequences.

Metalloproteinases, which require divalent cations such as zinc or calcium ions for the enzymatic activity or structural conformation, are widely distributed from bacteria to mammals. A variety of snake venom metalloproteinases has been isolated as the hemorrhagic factor, the specific activator, or the inhibitor of coagulation factors, or the inhibitor of platelet aggregation, etc. (*1–4*). Structural analysis of these snake venom metalloproteinases revealed that they are classified into four groups (P-I–P-IV) (*1, 2*). P-I is a group of ~25 kDa low-molecular mass metalloproteinases composed of only the metalloproteinase domain, and the P-II group has the disintegrin domain C-terminal to the metalloproteinase domain. P-III is a group of ~50 kDa high-molecular mass metalloproteinases containing the additional Cys-rich C-terminal domain. The P-IV group resembles P-III except for having an additional disulfide-linked C-type lectin subunit. These domain structures are also found in some membrane proteins called ADAMs (a disintegrin-like and metalloproteinase proteins) (*5*) or MDCs (metalloproteinase and disintegrin-like cysteine-rich proteins) participating in mammalian sperm–egg fusion (fertilin and cyritestin) (*6*), myotubule fusion (meltrin) (*7*), and *Drosophila* neurogenesis (KUZ) (*8*).

Disintegrins are snake venom peptides composed of fewer than 100 amino acid residues containing the Arg-Gly-Asp (RGD) sequence (*9–11*). They function as potent inhibitors of fibrinogen-dependent platelet aggregation induced by agonists such as collagen and ADP. The RGD sequence in a disulfide-linked loop structure is essential for eliciting the inhibitory effect since this sequence interferes with the ability of the platelet integrin $\alpha_{IIb}\beta_3$ [platelet membrane glycoprotein (GP)[1] IIb/IIIa] to interact with plasma fibrinogen (*12, 13*).

* To whom correspondence should be addressed: Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan. Telephone: (+81)-562-93-9381. Fax: (+81)-562-93-8832. E-mail: tmatsui@fujita-hu.ac.jp.
‡ Fujita Health University.
§ Fujita Health University College.
‖ Nara Medical University.

The disintegrins are synthesized in vivo as a portion of large precursor forms of metalloproteinases (P-II group) and processed by proteolysis (*2*). Interestingly, no snake venom metalloproteinases with the disintegrin-like domain (P-III group) contain the RGD sequence in the corresponding region, where the sequence is substituted with the X-X-Cys-Asp (XXCD) sequence. Although it was not certain whether this region possesses inhibitory activity for platelet aggregation, some synergistic effects have been proposed since P-III metalloproteinases are significantly more hemorrhagic than the P-I group. Usami et al. (*14*) first reported that jararhagin C, which contains only the disintegrin-like (Ser-Glu-Cys-Asp) and Cys-rich domains of jararhagin, has an inhibitory effect on collagen- and ADP-induced platelet aggregation. Recently, Zhou et al. (*15*) showed that a cyclic oligopeptide containing the SECD sequence of catrocollastatin in the P-III group inhibits collagen-induced platelet aggregation by binding to collagen. Other reports also suggested that the disintegrin-like sequences show an inhibitory activity to the agonist-induced platelet aggregation, such as disintegrins (*16−19*).

We recently purified a unique metalloproteinase, named kaouthiagin, from the venom of the cobra snake *Naja kaouthia* (*20*). Kaouthiagin is an *o*-phenanthroline or EDTA sensitive metalloproteinase which binds to and cleaves human von Willebrand factor (VWF) (*20, 21*). VWF is a multimeric plasma protein essential for platelet adhesion to the damaged subendothelial matrixes to form a hemostatic plug (*22*). Kaouthiagin cleaves VWF at a peptide bond between Pro708 and Asp709, resulting in a loss of the platelet- and collagen-binding activities of VWF by degrading the multimeric structure of VWF (*20*). In the paper presented here, we first determined the complete amino acid sequence of elapid venom metalloproteinase kaouthiagin and elucidated that it has a P-III type domain structure similar to those of viperid and crotalid metalloproteinases. Furthermore, we found that kaouthiagin has an extra disintegrin-like sequence in the Cys-rich domain which has an inhibitory effect on the agonist-induced platelet aggregation using synthetic peptides.

EXPERIMENTAL PROCEDURES

Materials. Kaouthiagin was purified from the crude venom powder of *N. kaouthia* obtained from the Japan Snake Institute (Gunma, Japan) as previously described (*20*). Normal citrated human plasma was obtained from healthy donors as previously described (*23*). *Achromobacter* protease I (AP-I) was a gift from T. Masaki (Ibaraki University, Ibaraki, Japan). *Pseudomonanas* aspartyl endopeptidase (Asp-N) and jack bean asparaginyl endopeptidase (Asn-C) were purchased from Takara Shuzo (Otsu, Japan). Hog stomach pepsin was from Koch-Light (Suffolk, England). Horseradish peroxidase (HRP)-conjugated anti-human VWF

was from Dakopatts (Glostrup, Denmark). Sialidase from *Arthrobacter ureafaciens* and recombinant peptide-*N*-glycosidase F (PNG-F) were from Nacalai Tesque (Kyoto, Japan) and Boehringer Mannheim Biochemica (Tutzing, Germany), respectively.

Reduction and S-Pyridylethylation. One milligram of kaouthiagin was reduced and S-pyridylethylated (PE) with tri-*n*-butylphosphine and 4-vinylpyridine in 0.3 M Tris-HCl buffer (pH 8.3) containing 6 M guanidine hydrochloride at 25 °C for 3 h as described previously (*24*). PE-kaouthiagin was purified by HPLC on a SynChropak RP-8 column (4.6 mm × 250 mm, SynChrom, Lafayette, IN) with a linear gradient of 0 to 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and concentrated by evaporation with a Speed Vac concentrator (Savant Instruments, Holbrook, NY).

Enzymatic and Chemical Cleavages of PE-Kaouthiagin. An aliquot of PE-kaouthiagin was subjected to CNBr cleavage (in 70% formic acid at 25 °C for 20 h) or proteolysis with AP-I which specifically cleaves lysyl bonds (*25*) [E:S ratio of 1:150 by mass, in 50 mM Tris-HCl buffer (pH 9.0) and 2 M urea at 37 °C for 6 h], Asp-N which specifically cleaves aspartyl bonds at the N-terminal side [E:S ratio of 1:100 by weight, in 50 mM sodium phosphate buffer (pH 8.0) at 37 °C for 18 h], or Asn-C which cleaves asparaginyl bonds at the C-terminal side [E:S ratio of 1:100 by weight, in 10 mM dithiothreitol, 1 mM EDTA, and 50 mM sodium acetate buffer (pH 5.0) at 37 °C for 24 h]. Several fragments were also subjected to secondary digestion with Asp-N or pepsin (E:S ratio of 1:100 by weight, in 10% acetic acid at 37 °C for 3 h). Peptides were separated by HPLC on a Cosmosil 5C18 column (4.6 mm × 150 mm, Nacalai Tesque) with gradients of acetonitrile into dilute aqueous TFA. Amino acid sequence determination was carried out with an Applied Biosystems Procise protein sequencing system (model 494 protein sequencer connected to a phenylthiohydantoin analyzer). The sequence similarities with other proteins were examined using the BLAST software program (*26*). In separate experiments, the intact kaouthiagin was subjected to CNBr cleavage (see below).

Mass Spectrometry. Mass determination was performed using an ion-spray mass spectrometer (PE-Sciex API-III biomolecular mass analyzer) and a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (PerSeptive Biosystems Voyager RPF). Approximately 5−30 pmol of peptides was dissolved in an appropriate volume of 33% 2-propanol containing 0.1% TFA and 50% acetonitrile and injected into the spectrometer for positive-mode analysis in ion-spray mass analysis. For MALDI-TOF MS, peptides (75−180 fmol) were mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid in 0.05% TFA and 50% acetonitrile, and analyzed in the linear mode with positive ion detection at an accelerating voltage of 20 kV with a mass accuracy of <0.1% Da.

Inactivation of Metalloproteinase Activity of Kaouthiagin. Kaouthiagin (400 µg/mL in TBS) was dialyzed against TBS containing 1 mM *o*-phenanthroline and 0.05% sodium azide for 22 h at 4 °C and against TBS alone for 4 h twice using a microdialyzer. Remaining *o*-phenanthroline content in the dialyzed sample was estimated by measuring the absorbance at 260 nm. VWF (16 µg) was incubated with intact, *o*-phenanthroline-treated kaouthiagin (10 µg/mL), or TBS at 37 °C for 0−60 min, and heated at 95 °C for 5 min after

addition of sodium dodecyl sulfate (SDS) buffer [final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol] and EDTA (final concentration of 10 mM). Aliquots (4 μg) were subjected to SDS−polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions. Protein concentrations were determined with the BCA reagent (Pierce, Rockford, IL) using BSA as a standard.

Glycosidase Digestion and CNBr Cleavage of Kaouthiagin. Kaouthiagin (10 μg) was preincubated with 0.5% SDS containing 10 mM EDTA at 95 °C for 2 min and digested with sialidase (10 milliunits) alone or sialidase (10 milliunits) plus recombinant PNG-F (1 unit) in the presence of 2% octyl glucoside and 40 mM phosphate buffer (pH 7.2) at 37 °C for 18 h. Digests were immediately frozen at −80 °C until they were used.

The intact kaouthiagin (0.4 nmol) was incubated with 0.6 μmol of CNBr in 70% formic acid at 25 °C for 25 h followed by evaporation to remove CNBr. Glycosidase- or CNBr-treated kaouthiagin was dissolved in a small amount of SDS buffer and subjected to SDS−PAGE.

SDS−PAGE. SDS−PAGE was performed using a 10 or 6% separation gel and a 5% upper gel according to the method of Laemmli (*27*) followed by protein staining using Coomassie brilliant blue or subjected to electroblotting.

VWF Binding Analysis. Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane using 10 mM CAPS buffer (pH 11.0) in 10% methanol (0.4 V/cm) at 4 °C for 1.3 h after SDS−PAGE (*28*). The membrane was soaked with TBS [150 mM NaCl and 20 mM Tris-HCl (pH 7.5)] containing 0.05% Tween 20 (Tw/TBS) overnight at 4 °C and incubated with normal human plasma (diluted 1:20 with Tw/TBS) for 1.5 h at room temperature. After the samples had been washed with Tw/TBS five times for 4 min, the membrane was incubated with HRP-conjugated anti-VWF antibody (diluted 1:2000 with Tw/TBS) for 1 h followed by HRP reaction using diaminobenzidine and H_2O_2 as described previously (*20*).

Synthetic Peptides. Cyclized peptides [RAAKHDCDL-PELC (D-1), CFDLNMRGDDGSFC (D-2), and CFDLN-MRGEDGSFC (D-3)] were custom-synthesized and air-oxidized at TANA Laboratories (Houston, TX) or Biologica Co. (Nagoya, Japan) and purified by HPLC on a SynChropak RP-18 column (4.6 mm × 250 mm, SynChrom) with a linear gradient of 0 to 60% acetonitrile containing 0.1% TFA in conjunction with sequencing analysis. Proper formation of the S−S linkage in the purified peptides was confirmed by comparing the mass before and after reduction with dithiothreitol by MALDI-TOF MS. Peptides were dissolved in dimethyl sulfoxide (DMSO) or 20 mM sodium acetate buffer (pH 5.0) before being used.

Platelet Aggregation Assay. Platelet-rich plasma (PRP) was prepared from freshly donated healthy normal blood containing 0.38% sodium citrate by gentle centrifugation (800 rpm for 10 min). PRP was mixed with a 15% volume of acid-dextrose solution (ACD, 75 mM sodium citrate, 38 mM citric acid, and 122 mM glucose) and centrifuged at 2300 rpm for 10 min. Pelleted platelets were washed with 10 mL of PBS [150 mM NaCl and 20 mM sodium phosphate buffer (pH 7.2)] containing 9 mM EDTA and 15% ACD and finally suspended in HEPES buffer [150 mM NaCl and 20 mM HEPES buffer (pH 7.35)] containing 1.8 mM $CaCl_2$ to obtain

3×10^5 platelets/μL. Platelets (300 μL) were preincubated with each peptide or kaouthiagin (1−3 μL) at 37 °C for 2 min with gentle stirring, and human fibrinogen (final concentration of 100 μg/mL) and agonists were successively added. Type I collagen (Horm-Chemie, Munchen, Germany, final concentration of 2 μg/mL) or ADP (final concentration of 10 μM) was added as an inducer for platelet aggregation. For ristocetin-induced platelet aggregation, PRP was used in the presence of ristocetin (final concentration of 1.6 mg/mL). Platelet aggregation was monitored by light transmittance change with a Chrono-Log aggregometer (Havertown, PA). Platelet aggregation induced by an agonist in the absence of peptides or kaouthiagin (addition of the same amounts of dissolving solvent) was expressed as 100% aggregation.

RESULTS

Primary Structure of Kaouthiagin. Analysis of the intact PE-kaouthiagin revealed the N-terminal sequence of 50 residues (Figure 1). Peptides obtained by limited proteolysis of PE-kaouthiagin with AP-I (prefix K), CNBr (prefix M), or Asp-N (prefix D) were fractionated by HPLC and subjected to sequencing analysis and mass spectrometry analysis (Table 1), resulting in provision of the overlapping sequences. CNBr cleavage of PE-kaouthiagin provided 10 peptides (M1−10, data not shown). M3 and M6 were large fragments and subjected to a second digestion with Asp-N, Asn-C (prefix N), or pepsin (prefix P). The AP-I digest provided 23 peptides (K1−23, data not shown), and K10 was subdigested with pepsin. The summary sequence is shown in Figure 1. Only one residue at position 112 gave no signal in the sequencing analysis. Since Thr was observed at residue 114, implying the presence of an Asn-linked sugar chain binding motif, it was assumed that this unknown residue is Asn. To examine the presence of the Asn-linked sugar chain, kaouthiagin was digested with recombinant PNG-F which specifically cleaves off Asn-linked sugar chains from glycoproteins. Intact kaouthiagin exhibited a relative molecular mass of ∼47 kDa, whereas the treatment with PNG-F reduced the molecular mass of kaouthiagin to ∼43 kDa (Figure 2A), indicating the presence of the Asn-linked sugar chain in kaouthiagin. Sialidase had no effect on the mobility of kaouthiagin, and the deglycosylated kaouthiagin exhibited a narrower band than it did before PNG-F digestion, suggesting that kaouthiagin has probably few or no sialic acid residues and that the broad band observed in the intact kaouthiagin preparation in SDS−PAGE is attributed to microheterogeneity of the Asn-linked sugar chain moiety.

Kaouthiagin consists of 401 amino acid residues (Figure 1). It has a zinc binding motif of HEXXHXXGXXH, which is conserved in the metzincin family, from residues 149 to 160 (Figure 3). A sequence comparison showed that kaouthiagin has similarities to P-III group snake venom metalloproteinases such as atrolysin A from *Crotalus atrox* (49%), HR1B (trimerelysin I) from *Trimeresurus flavoviridis* (47%), jararhagin (C) from *Bothrops jararaca* (50%), the H-I protease precursor from carpet viper (*Echis pyramidum leakeyi*) (49%), and the P-IV group of coagulation factor X activating enzyme heavy chain from Russel's viper (*Vipera russelli*) venom (47%). The metalloproteinase domain was 43−48% similar to P-I group low-molecular mass metallo-

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      1        10        20        30        40        50        60        70        80
      TNTPEQDRYLQAEKYIEFYVIVDNRMYRYYNYDKPAIKIRVYEMINAVNTKFRPLKIHIALIGLEIWSNEDKFEVKPAAS
      Intact-PE kaouthiagin                                                     M3-D27
      TNTPEQDRYLQAEKYIEFYVIVDNRMYRYYNYDKPAIKIRVYEMINAVNT--           DKFEVKPAAS
                                                 M3
                                                 INAVNTKFRPLKIHIALIGLEIWSNEDKFeVKP--


               90       100       110       120       130       140       150       160
      VTLKSFREWRQTVLLPRKRNDNAQLLTGINLNGTAVGIAYPGSLCTQRSVFVVQDYNRRMSLVASTMTHELGHNLGIHHD
      M3-D27(cont.)       K-10
      VTLKSFR--           RNDNAQLLTGINLxGTAVGIAYPGSLCTQRSVFVVQDYNRRMSLVAsT--
          K-9                                                          K10-P11
          SFREWRQTVLLPRK                                               VASTMTHELGHNLGIHHD
            M3-D14
            EWRQTVLLPRKRN


               170       180       190       200       210       220       230       240
      EASCICIPGPCIMLKKRTAPAFQFSSCSIRDYQEYLLRDRPQCILNKPLSTDIVSPAICGNYFVEEGEECDCGSPAACQS
      K10-P11 (cont.)                                           M6-D24
      EASCICIPGPCIMLK                                           DIVSPAICGNYFVEEGEEC
                    M6                                                M6-P15
                    LKKRTAPAFQFSSCSIRDYQEYLLRDRPQCILNKPLSTDIVspAI--  FVEEGEECDCGSPAACQS


               250       260       270       280       290       300       310       320
      ACCDAATCKFNGAGAECRAAKHDCDLPELCTGQSAECPTDSLQRNGHPCQNNQGYCYNGKCPTLTNQCIALLGPHFTVSP
      M6-P15(cont.)                             M6-D26                           M6-N17
      ACCDAATCKFNGAGAECRAAKHDCDL--             DSLQRNGHPCQNNQGYCYNGKCPT--        TVSP
                    K15                                            K-16
                    HDCDLPELCTGQSAECPTDSLQRNG--             CPTLTNQCIALLGPHFTVSP


               330       340       350       360       370       380       390       400
      KGCFDLNMRGDDGSFCRMEDGTKIPCAAKDVKCGRLYCTEKNTMSCLIPPNPDGIMAEPGTKCGDGMVCSKGQCVDVQTAY
      M6-N17 (cont.)     M8                   K21
      KGCFDL--          EDGTKIPCAAKDVK--      NTMSCLIPPNPDGIMAEPGTK
       K17                       D36                 M10-11
       GCFDLNMRGDDGSFCRMEDGTK    DVKCGRLYCTEKNTM--   AEPGTKCGDGMVCSKGQCVDVQTAY
      K16(cont.)
      K
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FIGURE 1: Amino acid sequence of kaouthiagin. Determined sequences of intact PE-kaouthiagin or peptides obtained by limited digestion with AP-I, CNBr, Asp-N, and Asn-C, designated with prefixes K, M, D, and N, respectively, were aligned below the summary sequence (bold). Some peptides were subdigested with Asp-N, Asn-C, or pepsin (P) after AP-I digestion or CNBr cleavage. Lowercase letters represent tentative identification, and an x represents an unidentified residue. Although the residue at position 112 gave no significant sequencer signal, this position was assumed to be an Asn (see the text). Products of lysyl and methionyl cleavage are numbered from the N-terminus toward the C-terminus of kaouthiagin. Products of pepsin, Asp-N, and Asn-C digestion were numbered in order of elution positions by HPLC.

proteinases (LHF-II, trimerelysin II, adamalysin II). Almost all the Cys residues (30 residues) are conserved, but 17 amino acid residues containing four Cys residues (between positions 249 and 250) are deleted in kaouthiagin (Figure 3). Kaouthiagin has the His-Asp-Cys-Asp sequence (residues 262−265) in the corresponding disintegrin-like domain. Furthermore, it uniquely contains the RGD sequence (residues 329−331) in the Cys-rich domain (Figure 3).

Effects of Inactivated Kaouthiagin and Synthetic Peptides of Disintegrin-like Domains on Agonist-Induced Platelet Aggregation. To study the effects of the disintegrin-like domain of kaouthiagin on platelet aggregation, the metalloproteinase activity was inactivated by *o*-phenanthroline treatment since there is a possibility that kaouthiagin might

have an affect on the platelets by its proteolytic activity. Kaouthiagin specifically cleaves VWF between Pro708 and Asp709 into two dimeric fragments of 350 and 220 kDa (Figure 2B) (*20*). The *o*-phenanthroline-treated kaouthiagin showed an inhibitory effect on neither the ristocetin-induced platelet aggregation nor the VWF-cleaving activity (Figure 2B) as previously described (*20*), but retained the VWF-binding activity (Figure 2A). This inactivated kaouthiagin exhibited an inhibitory effect on the collagen-induced platelet aggregation in a concentration-dependent manner (Figure 4A). Although it contained ∼0.2 mM *o*-phenanthroline after dialysis and the final concentration of *o*-phenanthroline in the experiments reached ∼7 μM, there were no significant effects of *o*-phenanthroline on the platelet aggregation up to

Table 1: Mass Spectrometric Analysis of PE-Kaouthiagin and Its Derived Peptides[a]

peptide	residue nos.	MH⁺ (Da) calculated	observed
intact PE-kaouthiagin	1–401	47616.0	ND[b]
K9	85–98	1816.0	1816 ± 0.6
K10	99–175	8754.3	ND[b]
K15	262–300	4821.1	4824 ± 1.1
K16	301–321	2450.3	2451 ± 0.6
K17	322–343	2664.0	2664 ± 0.0
K21	362–382	2291.1	2291 ± 0.4
M1	1–26	3187.6[c]	3187 ± 0.5
M2	27–44	2337.2[c]	2337 ± 0.0
K10-P11	143–175	3842.8	3847[d]

[a] PE-kaouthiagin and the peptides obtained by enzymic or chemical digestions were subjected to ion-spray MS or MALDI-TOF MS analysis. Values were calculated for MH⁺ from multiply charged signals that were observed. The molecular mass of each peptide was calculated as the monoisotopic mass from the deduced sequence. [b] Not determined. [c] MH⁺ was calculated as the peptide with homoserine lactone at the C-terminus. [d] MALDI-TOF MS analysis.

20 μM (data not shown). The IC$_{50}$ value of the *o*-phenanthroline-treated kaouthiagin was ~0.2 μM (Figure 5).

To further test the biological activities of the disintegrin-like domain and the RGD sequence found in the Cys-rich domain, we have examined the effects of synthetic peptides corresponding to the disintegrin-like sequences. Although the disulfide linkages were not determined, we designed simple loop peptides to form a single S–S bond. Peptides D-1 (RAAKHDCDLPELC) and D-2 (CFDLNMRGDDGSFC) located at residues 258–270 and 323–336, respectively, were synthesized and purified by HPLC. Peptide D-1 exhibited a single monoisotopic molecular mass (MH⁺) of 1467.8 (nonreduced form) and 1469.7 Da (reduced form) by MALDI-TOF MS, which were almost comparable to the calculated masses (1468.7 and 1470.7 Da, respectively). Peptide D-2 also exhibited MH⁺ of 1577.1 (nonreduced) and 1579.6 Da (reduced). These results indicated that the cysteinyl residues formed an intramolecular disulfide linkage in each peptide.

Washed platelets were preincubated with cyclized D-1 or D-2 peptide at various concentrations, and then fibrinogen and collagen were added to induce platelet aggregation. Cyclic peptide D-1 inhibited collagen-induced platelet aggregation with an IC$_{50}$ of ~90 μM (Figure 5), but the reduced linear peptide did not show an apparent inhibitory effect up to 330 μM. In the case of cyclic peptide D-2, it also inhibited the platelet aggregation in a concentration-dependent manner (Figure 4B). The IC$_{50}$ of D-2 was ~4.5 μM, which was ~20 times greater than that of cyclic D-1 but 20 times lower than that of *o*-phenanthroline-treated kaouthiagin (Figure 5). Cyclic peptide D-2 also inhibited ADP-induced platelet aggregation (data not shown). Cyclic peptide D-3, which had the same sequence as D-2 except that the RGD was substituted to the RGE sequence, had little effect on collagen-induced platelet aggregation up to 100 μM (Figure 5).

Effects of Deglycosylation and CNBr Cleavage on VWF-Binding Activity of Kaouthiagin. CNBr cleavage of PE-kaouthiagin provided several fragments (Figure 1), but only one large fragment with a molecular mass of ~43 kDa was observed after CNBr cleavage of the intact kaouthiagin
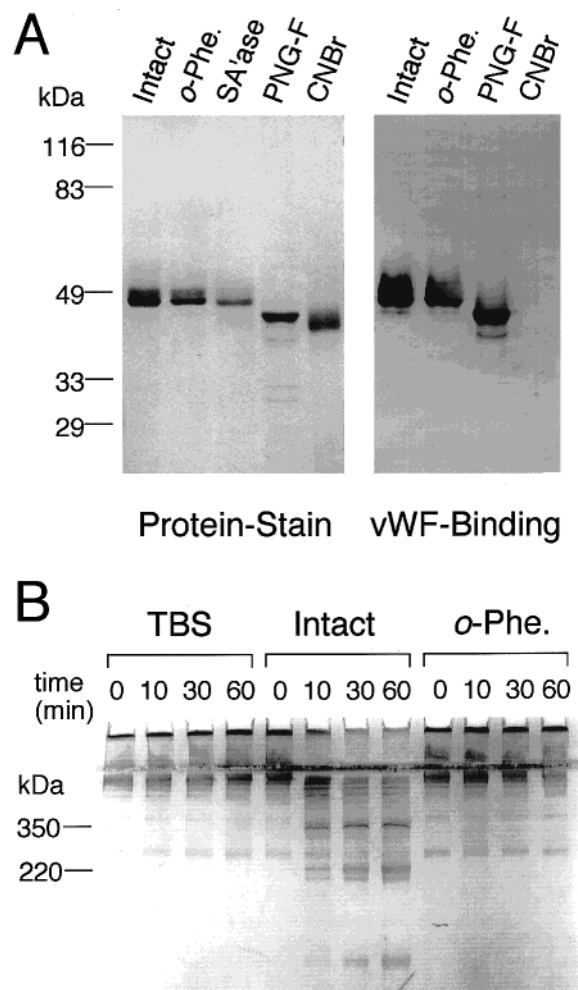

FIGURE 2: Effects of chemical modification on the VWF-binding and VWF-cleaving activities of kaouthiagin. (A) SDS–PAGE (left) and VWF-binding activity (right) of kaouthiagin after *o*-phenanthroline treatment, deglycosylation, and CNBr cleavage. Intact and modified kaouthiagins with *o*-phenanthroline (*o*-Phe), sialidase (SA′ase), sialidase with PNG-F, or CNBr [4 μg each except for sialidase digest (1 μg)] were subjected to SDS–PAGE under nonreducing conditions and protein-stained with Coomassie brilliant blue R-250 (left). Intact and modified kaouthiagins [0.4 μg each except for CNBr cleavage (4 μg)] as described above were electroblotted to a PVDF membrane after SDS–PAGE and incubated with human plasma (diluted 20-fold), followed by immunoblotting with the HRP-conjugated anti-VWF antibody (right). Numbers at the left indicate the molecular masses (kilodaltons) of marker proteins. (B) Effect of *o*-phenanthroline treatment on the vWF-cleaving activity of the kaouthiagin. VWF was incubated with TBS, intact, or *o*-phenanthroline-treated kaouthiagin (*o*-Phe.) for the indicated time, and aliquots (4 μg) were subjected to SDS–PAGE under nonreducing conditions. The intact VWF could not enter the 6% polyacrylamide gel, but the kaouthiagin-cleaved VWF exhibited ~350 and ~220 kDa bands.

(Figure 2A). N-Terminal sequence analysis of the large fragment indicated that it contains at least four N-termini starting at Ile45, Leu174, Glu339, and Ala377, demonstrating that it is composed of at least four fragments linked together by S–S linkages.

Kaouthiagin retained the VWF-binding activity even if it was deglycosylated by PNG-F digestion or *o*-phenanthroline treatment, but the activity was lost after CNBr treatment (Figure 2A). These results indicate that neither an Asn-linked sugar chain nor Zn²⁺ is essential for the VWF-binding activity of kaouthiagin, but the molecular conformation

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                         1        10        20        30        40        50        60        70        80        90       100       110
Kaouthiagin         TNTPEQDRYLQAEKYIEFYVIVDNRMYRYYNYDKPAIKIRVYEMINAVNTKFRPLKIHIALIGLEIWSNEDKFEVKPAASVTLKSFREWRQTVLLPRKRNDNAQLLTGINL
Atrolysin A              ERLTKRYVELVIVADHRMFTKYNGNLKKIRKWIYQIVNTINEIYIPLNIRVALVRLEIWSNGDLIDVTSAANVTLKSFGNWRVTNLLRRKSHDNAQLLTAIDL
RVV-X               LVSTSAQFNKIFIELVIIVDHSMAKKCN--STATNTKIYEIVNSANEIFNPLNIHVTLIGVEFWCDRDLINVTSSADETLNSFGEWRASDLMTRKSHDNALLFTDMRF
HR1B                QQRFPRRYIKLAIVVDHGIVTKHHGNLKKIRKWIYQLVNTINNIYRSLNILVALVYLEIWSKQNKITVQSASnVTLDLFGDWRESVLLKQRSHDCAQLLTTIDF
Jararhagin      149 TAEQQRY-DPYKYIEFFVVVDQGTVTKNNGDLDKIKARMYELANIVNEIFRYLYMHVALVGLEIWSNGDKITVKPDVDYTLNSFAEWRKTDLLTRKKHDNAQLLTAIDF
H-I             191 TSEQQR--SYKKYIEFVVVADYIMYRKYNNDSTAVRRRIYEIVNILNMVYIVFNIHVALTHIEIWSTRDQITVQSAADVTLDLFGDWRAKNLLTRKKHDNAQLFTGINL
LHF-II                   FSQKYIELVVVADHGMFTKYNGNLNTIRTRVHEIVNTLNGFYRSLNILISLTDLEIWSNQDLINVQSAANDTLKTFGEWRERVLLNRISHDNAQLLTAIDL
Trimerelysin II          QRFPQRYIELAIVVDHGMYKKYNQNSDKIKVRVHQMVNHINEMYRPLNIAISLNRLQIWSKKDLITVKSASNVTLESFGNWRETVLLKQQNNDCAHLLTATNL
Adamalysin II            QQNLPQRYIELVVVADRRVFMKYNSDLNIIRTRVHEIVNIINEFYRSLNIRVSLTDLEIWSGQDFITIQSSSSNTLNSFGEWRERVLLIWKRHDNAQLLTAINF


                         120       130       140       150       160       170       180        190       200       210
Kaouthiagin         nGTAVGIAYPGSLC-TQRSVFVVQDYNRRMSLVASTMTHELGHNLGIHHDEA-SCICIPGPCIMLKK-RTAPAFQFSSCSIRDYQEYLLRDRPQCILNKPLSTDIVSPAIC
Atrolysin A         DEETLGLAPLGTMCDPKLSIGIVQDHSPINLLVAVTMAHELGHNLGMVHDEN-RCHCSTPACVMCAVLRQRPSYEFSDCSLNHYRTFIINYNPQCILNEPLQTDIISPPVC
RVV-X               DLNTLGITFLAGMCQAYRSVEIVQEQGNRNFKTAVIMAHELSHNLGMYHDGK-NCICNDSSCVMSPVLSDQPSKLFSNCSIHDYQRYLTRYKPKCIFNPPLRKDIVSPPVC
HR1B                DGPTIGKAYTASMCDPKRSVGIVQDYSPINLVVAVIMTHEMGHNLGIPHDGN-SCTCGGFPCIMSPMISDPPSELFSnCSKAYYQTFLTDHKPQCILNAPSKTDIVSPPVC
Jararhagin          NGPTIGYAYIGSMCHPKRSVGIVQDYSPINLVVAVIMAHEMGHNLGIHHDTG-SCSCGDYPCIMGPTISNEPSKFFSNCSYIQCWDFIMNHNPECIINEPLGTDIISPPVC
H-I                 NGQTLGIARMRGMCSPSNSVGVIQDYCKNYLLVAITMAHELGHNLGMDHDNG-NCNCPDTSCIMSAVAGPEPVFSFSNCSRNDYRSFRNSDQSKCIDNKPLKTDIVSPSVC
LHF-II              ADNTIGIAYTGGMCYPKNSVGIVQDHSPKTLLIAVTMAHELGHNLGMKHDEN-HCHCSASFCIMPPSISEGPSYEFSDCSKDYYQMFLTKRKPQCILNKP
Trimerelysin II     NDNTIGLAYKKGMCNPKLSVGLVQDYSPNVFMVAVTMTHELGHNLGMEHDDKDKCKC--EACIMSDVISDKPSKLFSDCSKNDYQTFLTKYNPQCILNAP
Adamalysin II       EGKIIGKAYTSSMCNPRSSVGIVKDHSPINLLVAVTMAHELGHNLGMEHDGKD-CLRGASLCIMRPGLTPGRSYEFSDDSMGYYQKFLNQYKPQCILNKP


                         220       230       240                  250       260       270       280       290       300       310
Kaouthiagin         GNYFVEEGEECDCGSPAACQSACCDAATCK----------------FNGAGAECRAAK--HDCDLPELCTGQSAECPTDSLQRNGHPCQNNQGYCYNGKCPTLTNQCIAL
Atrolysin A         GNELLEVGEECDCGSPRTCRDPCCDAATCKLHSWVECESGECCQQCKFTSAGNVCRPAR--SECDIAESCTGQSADCPTDDFHRNGKPCLHNFGYCYNGNCPIMYHQCYAL
RVV-X               GNEIWEEGEECDCGSPANCQNPCCDAATCKLKPGAECGNGLCCYQCKIKTAGTVCRTRRARDECDVPEHCTGQSAECPRDQLQQNGKPCQNNRGYCYNGDCPIMRNQCISL
HR1B                GNELLEAGEECDCGSPENCQYQCCDAASCKLHSWVKCESGECCDQCRFRTAGTECRAAE--SECDIPESCTGQSADCPTDRFHRNGQPCLYNHGYCYNGKCPIMFYQCYFL
Jararhagin          GNELLEVGEECDCGTPENCQNECCDAATCKLKSGSQCGHGDCCEQCKFSKSGTECRASM--SECDPAEHCTGQSSECPADVFHKNGQPCLDNYGYCYNGNCPIMYHQCYAL
H-I                 GNYFVEVGEECDCGSRTYCRNPCCNATTCKLTPGSQCADGECCNQCRFRPARTECRRKI--DDCDVPEYCTGQSGECPLDVFQRNGQPYQSNNGYCYNGNCPILKNQCIHL


                         320       330       340       350       360       370       380       390       400
Kaouthiagin         LGP-----HFTVSPKGCFDLNMRGDDGSFCRMEDGTKIPCAAKDVKCGRLYCTEKNTMS---CLIPPNPDGI---MAEPGTKCGDGMVC-SKGQCVDVQTAY
Atrolysin A         WGS----NVTVAPDACFDINQSGNNSFYCRKENGVNIPCAQEDVKCGRLFC-NVNDFL---CRHKYSDD----GMVDHGTKCADGKVC-KNRQCVDVTTAYKSTSGFSQI
RVV-X               FGS----RANVAKDSCFQENLKGSYYGYCRKENGRKIPCAPQDVKCGRLFCLNNSPRNKNPCNMHYSCMDQHKGMVDPGTKCEDGKVCNNKRQCVDVNTAYQSTTG
HR1B                FGS----nATVAEDDCFNNNKKGDKYFYCRKENEKYIPCAQEDVKCGRLFCDNKKY----PCHYnYSE-DLDFGMVDHGTKCADGKVC-SNRQCVDVNEAYKS
Jararhagin          FGA----DVYEAEDSCFKDNQKGNYYGYCRKENGKKIPCAPEDVKCGRLYCKDNSPGQNNPCKMFYSNDDEHKGMVLPGTKCADGKVC-SNGHCVDVATAY
H-I                 WKPAPPAGVNVAPDVCFEDNQNGTDVYNCGIKNGTYIKCARQDIKCGRLSCEEPSTGNTINCQAFFSSRNPDYGMVDIGTKCADGKVCNSNRHCVDVNTAY
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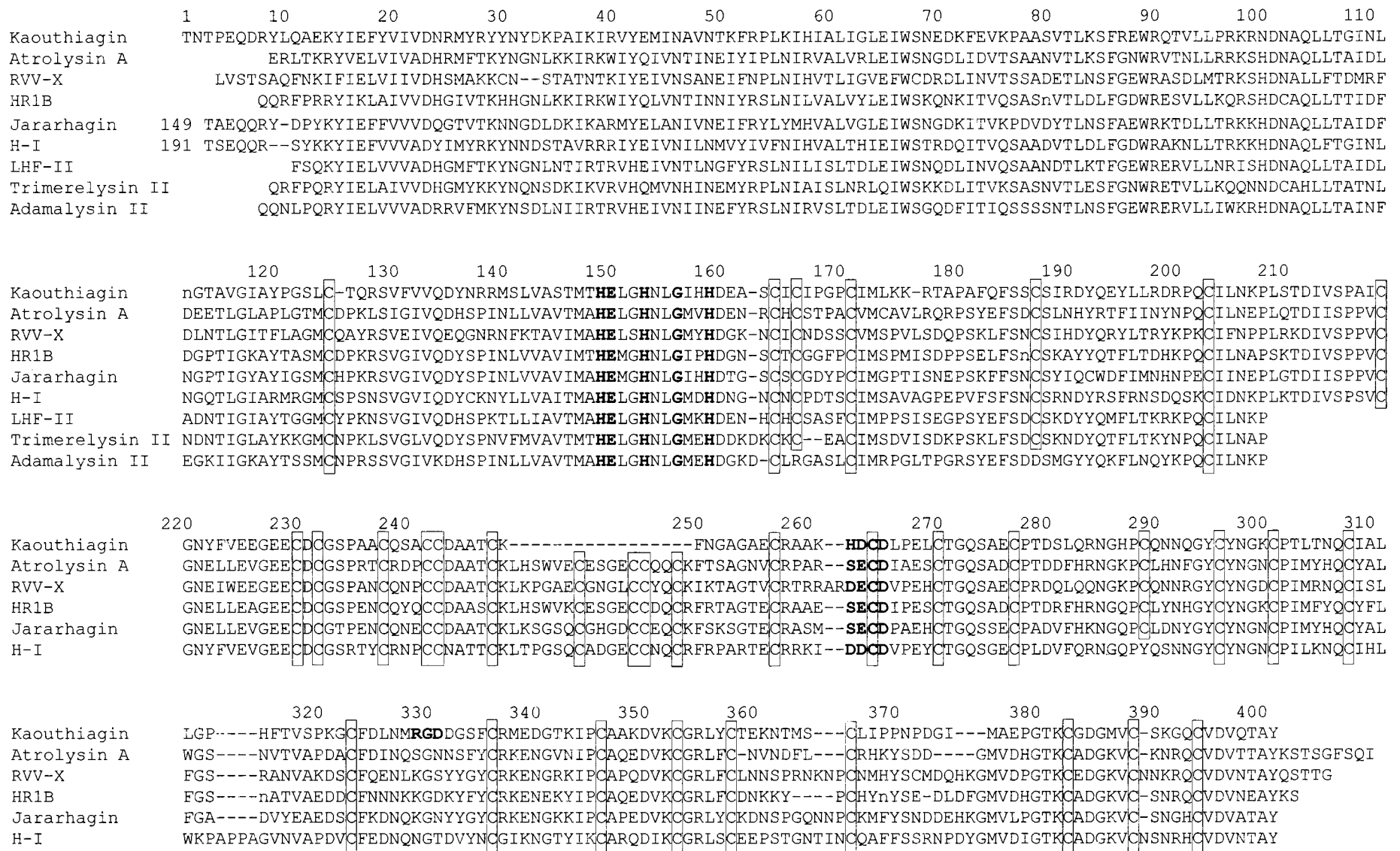
FIGURE 3: Comparison of the amino acid sequence of kaouthiagin with those of other snake venom metalloproteinases. The amino acid sequence of kaouthiagin was aligned with those of other snake venom metalloproteinases, atrolysin A from *C. atrox* (*35*), RVV-X (coagulation factor X activating enzyme from Russel's viper venom) (*38*), HR1B (trimerelysin I) from *T. flavoviridis* (*36*), jararhagin (C) from *B. jararaca* (*34*), H-I protease from carpet viper (*39*), LHF-II from *Lachesis muta muta* (*40*), trimerelysin II from *T. flavoviridis* (*41*), and adamalysin II from *Crotalus adamanteus* (*42*). Gaps were inserted to obtain maximum degrees of similarity. Lowercase letters indicate the tentative glycosylation sites. Zinc binding motif (HEXXHXXGXXH), disintegrin-like (XXCD), and RGD sequences in kaouthiagin are expressed in boldface. Conserved Cys residues are boxed. Numbers on the top indicate the residue number of kaouthiagin.
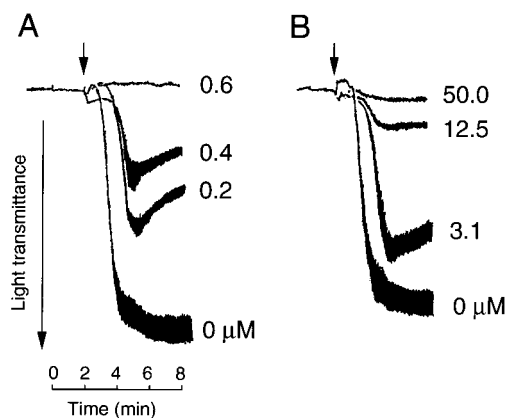
FIGURE 4: Effects of *o*-phenanthroline-treated kaouthiagin and synthetic peptides on platelet aggregation. (A) Washed platelets (3 × 10^5 platelets/μL) were preincubated with *o*-phenanthroline-treated kaouthiagin (final concentrations of 0.2, 0.4, and 0.6 μM) for 2 min at 37 °C, and then fibrinogen (final concentration of 100 μg/mL) and collagen (final concentration of 2 μg/mL) were added to induce platelet aggregation at the time as shown by arrows. The same aliquot of TBS alone was added as a control (0 μM). Aggregation was monitored as light transmittance with an aggregometer. (B) Washed platelets were preincubated for 2 min at 37 °C in the presence of various concentrations of cyclized peptide D-2 (CFDLNMRGDDGSFC) dissolved in DMSO (final concentrations of 3.1, 12.5, and 50 μM), and then fibrinogen and collagen were added as shown in panel A. The same aliquot of DMSO alone was added as a control (0 μM).
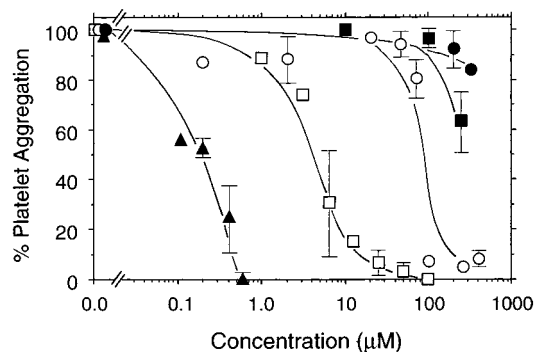
FIGURE 5: Inhibition of collagen-induced platelet aggregation by *o*-phenanthroline-treated kaouthiagin and synthetic peptides. Washed platelets (3 × 10^5 platelets/μL) were preincubated with various concentrations of *o*-phenanthroline-treated kaouthiagin (▲), synthetic peptide D-1 (○) (RAAKHDCDLPELC), reduced D-1 (●), D-2 (□) (CFDLNMRGDDGSFC), and D-3 (■) (CFDLNM-RGEDGSFC) for 2 min at 37 °C, and then fibrinogen (final concentration of 100 μg/mL) and collagen (final concentration of 2 μg/mL) were added to induce platelet aggregation. Results were represented as percentage of control values using the same amounts of TBS (for *o*-phenanthroline-treated kaouthiagin), 20 mM acetate buffer, pH 5.0 (for peptide D-1) and DMSO (for peptides D-2 and D-3) alone. The data were obtained using platelets from three different donors.

disrupted by CNBr cleavage in addition to reduction of the S−S linkages (*20*) is critical for the VWF-binding activity.

DISCUSSION

In the study presented here, we first determined the complete amino acid sequence of elapid venom metalloproteinase kaouthiagin. The sequence indicated that kaouthiagin is classified into the high-molecular mass metalloproteinase P-III group comprised of N-terminal metalloproteinase,

disintegrin-like, and C-terminal Cys-rich domains, such as crotalid and viperid venom metalloproteinases (*1*, *2*). It contained a typical Zn^{2+}-binding motif conserved in the metzincin family (*29*) and was in agreement with the results that kaouthiagin lost its proteolytic activity by treatment with *o*-phenanthroline or EDTA as previously described (*20*). The disintegrin-like domain showed the HDCD sequence at the position corresponding to the RGD sequence of disintegrins. This histidyl-aspartyl sequence has not yet been reported in disintegrin-like sequences in viperids and crotalids. Since kaouthiagin exhibited a unique deletion of 17 residues containing four Cys residues in the vicinity of the disintegrin-like domain, it was assumed that this deletion might have an effect on the conformation of the disintegrin-like domain (*30*). One of most interesting features found in the kaouthiagin sequence is the presence of the RGD sequence (residues 329−331) in the Cys-rich domain. It is located in the middle of 12 residues between two Cys residues. This location is similar to the disintegrin and disintegrin-like sequences containing 11 and 12 residues between two Cys residues, respectively.

The biological function of the disintegrin-like and Cys-rich domains has not been clearly elucidated. In jararhagin from *B. jararaca* venom, the disintegrin-like and Cys-rich domain (named jararhagin C) exhibited an inhibitory activity toward the platelet aggregation induced by collagen and ADP (*14*). Kamiguti et al. (*31*) suggested that jararhagin bound to the $\alpha_2 I$ domain of platelets by its disintegrin-like domain and then hydrolyzed the β_1 subunit by its metalloproteinase domain. However, Ivaska et al. (*32*) showed that a synthetic cyclized peptide corresponding to the jararhagin disintegrin-like sequence had no inhibitory effect, but another sequence (Arg-Lys-Lys motif) in the metalloproteinase domain had a direct binding activity to the collagen receptor of the platelet integrin $\alpha_2 I$ domain. Jia et al. (*30*) indicated that the native or insect cell-expressed recombinant disintegrin-like plus Cys-rich domains of atrolysin A from *C. atrox*, which have the SECD sequence in the disintegrin-like domain as jararhagin (C) does, inhibited both collagen- and ADP-induced platelet aggregation with IC₅₀ values of 0.1−0.5 μM. They also showed that synthetic loop peptides [CR-PARSECDIAESC-acetoamidomethyl (Acm) and Acm-CR-PARSECDIAESC] had an inhibitory activity against collagen-induced aggregation with an IC₅₀ of 200−400 μM, which was 500 times lower than those of the native or recombinant proteins. The same results were presented by Shimokawa et al. (*17*) in catrocollastatin C which is also comprised of the disintegrin-like and Cys-rich domains. They used an extremely high concentration (1−1.5 mM) of the disulfide-linked synthetic peptide (Acm-CRASMSECDPAEHC-NH₂), when compared to that of native catrocollastatin C (IC₅₀ = 66 nM), to partially inhibit collagen- and ADP-induced platelet aggregation. These results seem to imply that peptides simply cyclized by adjacent Cys residues might result in an improper conformation around the disintegrin-like sequence to elicit the full activity, because adjacent Cys residues flanking the RGD sequence do not form the disulfide bond together in native disintegrins (*11*). The exact disulfide linkages have not yet been elucidated for these proteins. Another implication might be that other parts of the domain

synergistically facilitate the effect of the disintegrin-like domain. Recently, Jia et al. (*33*) indicated that the recombinant Cys-rich domain of atrolysin A has an ability to inhibit collagen-induced platelet aggregation. In kaouthiagin, as we present in this study, the second disintegrin-like RGD sequence was found in the Cys-rich domain. In a preliminary experiment, kaouthiagin inhibited collagen-induced platelet aggregation. Since ADP- or collagen-induced platelet aggregation is dependent on GPIIb/IIIa and fibrinogen, whereas kaouthiagin interacts with neither fibrinogen nor GPIIb/IIIa (*20*), these results suggest that kaouthiagin has a disintegrin-like activity like jararhagin C (*14*) or cleaves collagen receptors on platelets such as $\alpha_2\beta_1$ integrinlike jararhagin (*31*). In the study presented here, the metalloproteinase-inactivated kaouthiagin still exhibited an inhibitory effect on collagen-induced platelet aggregation (Figure 4A). We then focused on the effects of the disintegrin-like domain and the RGD sequence found in the Cys-rich domain and designed a cyclic peptide for each. Although there is a possibility for other kinds of formations of disulfide linkages in the disintegrin-like domain, only one cyclized form with a Cys264−Cys270 linkage (D-1) was tested. Cyclic peptide D-1 had an inhibitory effect on platelet aggregation, but the linear peptide did not, suggesting the importance of the S−S linkage to the inhibitory activity of D-1. There is a possibility that an alternate S−S linkage would provide it with more potency than D-1 has. Peptide D-2 containing the RGD sequence in the middle of the Cys323−Cys336-linked loop strongly inhibited the platelet aggregation stimulated by collagen with an IC_{50} value of ∼4.5 μM. Control peptide D-3 having the RGE sequence instead of the RGD sequence of peptide D-2 had little effect at the same concentration, indicating the importance of the RGD sequence. Although we have no direct information about the correct conformation of the disintegrin-like and Cys-rich domains in kaouthiagin, the inhibitory effect on platelet aggregation observed with kaouthiagin might be attributable to both the disintegrin-like domain and the RGD sequence in the Cys-rich domain.

Other P-III group snake venom proteinases also have conserved Cys residues assigned to Cys323 and Cys336 of kaouthiagin (Figure 3). The corresponding RGD sequence is substituted with KGN, SGN, and KGD sequences in the Cys-rich domain of jararhagin (*34*), atrolysin A (*35*), and HR1B (*36*), respectively. It is noteworthy that the disintegrin from *Sistrurus miliarius barbouri* (barbourin) contains the KGD sequence instead of the RGD sequence (*37*). These results suggest a possibility that the corresponding part of the Cys-rich domain in other P-III group snake venom metalloproteinases also has a synergistic disturbing activity for platelet aggregation, serving as a second disintegrin-like domain. More experiments using synthetic peptides or recombinant proteins with modified amino acid residues at the corresponding position seem to be necessary for elucidating the function of the Cys-rich domains in P-III group snake venom metalloproteinases.

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