Determination of the structure of two novel echistatin variants and comparison of the ability of echistatin variants to inhibit aggregation of platelets from different species

Yuh-Ling CHEN,* Tur-Fu HUANG,† Shun-Wen CHEN* and Inn-Ho TSAI*‡

*Institute of Biological Chemistry, Academia Sinica, P.O. Box 23-106, Taipei, Taiwan, Republic of China and †Department of Pharmacology, National Taiwan University, Taipei, Taiwan, Republic of China

Two new variants of short disintegrins were purified from the venom of *Echis carinatus leakeyi* and named echistatin β and γ . These proteins were found to be about 85% similar in amino acid sequence to echistatin α which has been well studied. The disulphide pattern of echistatin γ appeared to be identical with that of echistatin α . They all contain the adhesive recognition sequence Arg-Gly-Asp (RGD) but inhibit the aggregation of platelets from human and other mammals with different potencies. Echistatin β and α are far more effective on platelets from humans and guinea pigs than those from rabbits and rats whereas echistatin γ is less discriminating of the platelets of the species tested. This species-dependent platelet sensitivity to echistatin β and γ could be attributed to the variations in

residues 15, 21, 22 and 27, which are close to or within the RGD loop, rather than to the C-terminal variations after residue 46. Taking advantage of the presence of methionine residues flanking both sides of the ARGDDM motif in echistatin γ , we deleted this hexapeptide by CNBr cleavage to produce des-(23–28)-echistatin γ . The modified protein showed c.d. and fluorescent spectra grossly similar to the intact echistatin but its antiplatelet potency decreased more than 200-fold. We thus propose that a favourable conformation of the RGD region is responsible mainly for the high-affinity binding of echistatin to the platelet glycoprotein IIb–IIIa as shown previously for the binding of medium-size disintegrin.

INTRODUCTION

A family of proteins isolated from snake venoms and leeches have been designated as disintegrins because they antagonize the activity of integrins [1–4]. Binding to the glycoprotein IIb–IIIa on platelet membranes and inhibiting the binding of fibrinogen, they are potent platelet-aggregation inhibitors [5,6]. A recent study [7] has demonstrated that triflavin (a member of the disintegrin family) binds to glycoprotein IIIa of activated platelets. The homologous and cysteine-rich snake venom proteins contain the Arg-Gly-Asp (RGD) sequence except for barbourin which has the Lys-Gly-Asp (KGD) sequence instead [8]. The RGD or KGD motif is involved in the binding of fibrinogen [9], vitronectin [10] and fibronectin [11] to their receptors. Thus disintegrins are potential antithrombotic agents [12] able to interfere with the integrin-mediated events. For example, albolabrin and triflavin significantly inhibited the metastasis of B16 cells in mouse lungs [2,6], and it was reported that echistatin bound to the α_v -like integrin of osteoclasts and inhibited bone resorption by osteoclasts [13,14].

Echistatin α_1 , a 49-amino acid disintegrin, was isolated from the venom of the sand viper *Echis carinatus* [15], and the variant echistatin α_2 , which is two residues shorter than α_1 at the C-terminus, was found [1]. However, a platelet-aggregation inhibitor called carinatin was previously purified from the same viper venom [16] but its isoelectric point (4.8) was different from those reported for echistatin α_1 and α_2 (8.3). Therefore we

speculated that echistatin variants other than α might exist in subspecies of *E. carinatus*. Characterization of venom disintegrins from closely related species may promote a better understanding of the structure and function relationships of this family of proteins. In the present work we purified two novel variants of echistatin, which we named echistatin β and γ , from the venom of *Echis carinatus leakeyi*, and determined their complete amino acid sequences, and also the disulphide bridge pattern of echistatin γ . We also examined the effect of chemical modifications, such as oxidation of methionine residues flanking the RGD motif and removal of the RGD region by CNBr cleavage, on the antiplatelet activity of echistatin β and γ . The significance of the presence of more than one disintegrin in one venom source and their structure–function relationships were investigated.

MATERIALS AND METHODS

Materials

E. carinatus leakeyi venom was purchased from Ventoxin Laboratories (Frederick, M.D., U.S.A.). Sephadex G-75 was from Pharmacia LKB. Trifluoroacetic acid (TFA), acetonitrile, and CNBr were purchased from Merck. Echistatin α_1 , ADP, apyrase, Trizma base, sodium citrate, *N*-acetyl-L-methionine, chloramine-T and Arg-C endopeptidase were obtained from Sigma. Phenyl isothiocyanate was purchased from Applied Biosystems. Endoproteinase Asp-N was from Boehringer, Mannheim, Germany. 4-*NN*-Dimethylaminoazobenzene-4'-iodoacet-

Abbreviations used: FITC, fluorescein isothiocyanate; PRP, platelet-rich plasma; PTH, phenylthiohydantoin; Rn, rhodostomin; SCM, S-carboxymethyl; S-DABCAM, 4-dimethylaminoazobenzene-4'-carboxyamidomethyl; S-DABIA, 4-*NN*-dimethylaminoazobenzene-4'-iodoacetamino-2'-sulphonic acid; TFA, trifluoroacetic acid.

‡ To whom correspondence should be addressed.

amido-2'-sulphonic acid (S-DABIA) was a generous gift from Dr. J.-Y. Chang through Dr. C. S. Liu [17]. Des-(46-49)-echistatin γ was synthesized on the basis of our sequence data by Dr. S. H. Wu and Dr. K. T. Wang using a peptide synthesizer (model 431A, Applied Biosystems).

Platelet-aggregation assay

Blood was collected from rabbits, Sprague-Dawley rats, guinea pigs or healthy human donors and mixed with 3.6% sodium citrate (9:1, v/v) before centrifugation at 200 *g* for 10 min at 25 °C, to prepare the platelet-rich plasma (PRP) [18]. Platelet aggregation was measured by the turbidimetric method [19] using an aggregometer (Jasco CAF-100 Ca²⁺ analyser). The disintegrin samples were added to PRP 1 min before initiation of the aggregation by 10 μ M ADP. The IC₅₀ value of a disintegrin, defined as the concentration to inhibit 50% of the aggregation of PRP caused by 10 μ M ADP, was determined from the dose-dependence curve.

Purification of echistatin β and γ

About 250 mg of the crude venom of *E. carinatus leakeyi* was dissolved in 1.5 ml of ammonium acetate, pH 6.8, and loaded on a Sephadex G-75 column. The column was eluted at a flow rate of 0.25 ml/min and fractions of 2.5 ml were collected. The fractions containing platelet-aggregation-inhibitory activity were pooled and purified by reversed-phase h.p.l.c. on a column (4.6 mm $\times$ 250 mm) of C18 gel (ODS-H; 5 μ m; Chemcosorb Scientific Co., Osaka, Japan) using a gradient of solution B (0.07% TFA in acetonitrile). The column was eluted at 1 ml/min under isocratic conditions (7% solution B) for the first 5 min followed by a 35 min linear gradient up to 27% solution B. Rabbit PRP was used to assay the aggregation inhibitor. The purity of echistatin β and γ after h.p.l.c. was assessed by SDS/PAGE and automated N-terminal sequencing.

Protein and amino acid analysis

Protein concentration was determined spectrophotometrically at 205 nm by using the following equation: concentration (mg/ml) = $A_{205}/31$ [20]. The concentration of echistatin was also checked by amino acid analysis after vapour-phase hydrolysis at 158 °C for 30 min using 7 M HCl/10% TFA/0.1% phenol (by vol.) [21,22]. Amino acids in the hydrolysate were derivatized to dimethyl aminoazobenzenesulphonyl amino acids before separation by reversed-phase h.p.l.c. [23]. The hydrolysate of purified egg white lysozyme were used as a reference.

Endoprotease and CNBr treatment and sequencing

Echistatin (200 μ g) was reduced in 100 μ l of 0.2 M Tris/HCl (pH 8.0) containing 6 M guanidinium chloride and 20 mM dithiothreitol and incubated at 37 °C for 2 h. After the addition of 10 μ l of 2M iodoacetic acid and incubation at 37 °C for 20 min in the dark, the S-carboxymethylated (SCM-) proteins were isolated by reversed-phase h.p.l.c., and subjected to protein sequencing.

The SCM-echistatin (50 μ g) was hydrolysed with 1 μ g of Arg-C endopeptidase in 0.1 ml of 50 mM Tris/HCl containing 1 mM CaCl₂ and 5 mM dithiothreitol (pH 7.5), for 20 h at 37 °C. Alternatively, for the CNBr-cleavage reaction, it was solubilized in 100 μ l of 65% formic acid containing 0.1 mg of CNBr in the dark for 20 h at room temperature. After h.p.l.c. the cleaved products were subjected to amino acid analysis and sequencing. An automated gas-phase sequencer (model 477A, Applied Biosystems) was used, and the phenylthiohydantoin (PTH) amino

Figure 1 Gel-permeation chromatography of *E. carinatus leakeyi* venom

The crude venom (0.25 g in 1.5 ml of buffer) was applied to a column (1.5 cm $\times$ 95 cm) of Sephadex G-75 which was equilibrated and eluted with 50 mM ammonium acetate, pH 6.8, at 4 °C. The peak containing platelet-aggregation inhibitor is indicated by an arrow.

acid derivatives were determined by an on-line h.p.l.c. system [24].

C-terminal analysis

Carboxymethylated proteins (5 μ g) were digested with 0.5 μ g of carboxypeptidase Y in 0.1 M ammonium acetate, pH 6.0, at room temperature [25]. After different lengths of time, samples were removed, 0.1 mM di-isopropyl fluorophosphate was added to stop the reaction, and the samples were dried in vacuum. The released residues were derivatized to dimethylaminoazobenzene-sulphonyl amino acids and analysed [23].

Identification of disulphide bridges in echistatin γ

Echistatin γ (50 μ g) was digested with 1 μ g of endoproteinase Asp-N in 100 μ l of 0.05 M sodium phosphate buffer, pH 8.0, for 16 h at 37 °C. The digested fragments were separated by reversed-phase h.p.l.c. using a Chemcosorb ODS-H column and a linear gradient from 5 to 30% acetonitrile in 0.07% TFA. By automated protein sequencing we identified a fragment (R2, Figure 5a) containing the polypeptides 1–25 and 30–49 linked by four disulphide bridges. The stepwise degraded PTH-amino acid derivatives of R2 at the fifth, seventh and eighth cycle were recovered, and each was reduced with 0.5 nmol of dithiothreitol in 20 μ l of 0.1 M NH₄HCO₃ buffer (pH 8.5) for 1 h at room temperature, and then 10 nmol of S-DABIA [17] in 2 μ l of water was added. The mixture was incubated for another hour at room temperature in the dark and 20 μ l of 1% TFA were added before purification by reversed-phase h.p.l.c. The synthetic 4-dimethylaminoazobenzene-4'-carboxyamidomethyl derivative of PTH-cysteine (S-DABCAM PTH-Cys) was prepared and used as the h.p.l.c. standard [26].

Oxidation of methionine residues

Echistatin β and γ (20 μ g) were dissolved in 200 μ l of 0.1 M borate buffer, pH 8.5, and oxidized with up to 160 μ M chloramine-T [27]. After incubation for 30 min at 25 °C, the excess oxidant was scavenged by 200 μ M N-acetylmethionine. Aggregation-inhibition activity of the modified proteins was studied using the rabbit and human PRP.

C.d. spectroscopy

C.d. studies were performed with a Jasco J-720 spectropolarimeter under nitrogen gas. The spectra were measured in a thermostatically controlled 1 mm quartz cuvette at a peptide concentration of 50 μ M in water (pH $\approx$ 5) with 0.5 nm band width, 0.2 nm step resolution and a 2.0 s time constant. For each spectrum, three scans were averaged, corrected for solvent contributions and analysed by non-linear regression.

Fluorescein isothiocyanate (FITC) conjugation of rhodostomin (Rn)

Rn was purified from *Calloselasma rhodostoma* venom (Latoxan) as described previously [28]. FITC solution freshly prepared before the conjugation reaction (10 μ l) was added to Rn stock solution (6–8 mg/ml in 0.1 M NaHCO₃) and incubated at room temperature for 1 h with continuous vortexing. The freshly prepared 1.5 M hydroxylamine (pH 8.0–8.5) was then added to stop the reaction, and a Sephadex G-10 column equilibrated with PBS was used to isolate FITC-conjugated rhodostomin (FITC-Rn) from the mixture. The antiplatelet activity of Rn was not affected by the labelling reaction.

Flow-cytometry measurement

Venous blood from an adult donor who had taken no medicine for 2 weeks was collected and centrifuged to obtain PRP and its platelet count was adjusted to 2×10^8 platelets/ml with saline. PRP (20 μ l) and FITC-Rn (2 μ l) were added to polypropylene tubes containing 178 μ l of Tyrode solution (0.4 mM NaH₂PO₄, 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO₃, 0.5 mM CaCl₂ and 3.5 mg/ml BSA, pH 7.4) and mixed. After 30 min at room temperature, the mixture was brought to 2 ml with Tyrode solution and immediately subjected to fluorescent flow-cytometer analysis. For displacement experiments, native or des-(23–28)-echistatin γ was added 3 min before the addition of FITC-Rn. Non-specific binding of FITC-Rn was determined using EDTA/Tyrode solution (5 mM EDTA and no Ca²⁺) instead of Tyrode solution. A Coulter Epics Profile flow cytometer (Coulter Electronics) was used to analyse the fluorescence histograms. Excitation and emission wavelengths were at 488 nm and 525 nm respectively. Each histogram was generated by quantifying the fluorescence intensity of 50 000 platelets, and the peak height was indicative of the number of platelets with a given intensity.

RESULTS

Purification of echistatin β and γ

Echistatin variants were purified to homogeneity from the venom of *E. carinatus leakeyi* in two steps: first gel-filtration chromatography (Figure 1), then reversed-phase h.p.l.c. (Figure 2). Two active inhibitors were eluted at 13 and 23% acetonitrile and designated echistatin β and γ respectively. Their respective yields were about 0.5 and 1% (w/w) of the venom. Echistatin α was not found in this venom.

Amino acid sequences

The amino acid sequences of echistatin β and γ were determined by automated sequencing of their SCM derivatives up to the 46th residue, as well as the peptides derived by CNBr cleavage and endoproteinase Arg-C digestion. The complete amino acid sequences of four echistatin variants are listed for comparison (Figure 3). Their C-terminal sequences were also confirmed by

Figure 2 Reversed-phase h.p.l.c. purification of echistatins

A Chemcosorb ODS-H column was equilibrated in 7% acetonitrile containing 0.07% TFA before the active material from Sephadex G-75 was loaded. The column was eluted at 1 ml/min with a linear gradient of acetonitrile over 40 min. Peaks containing echistatin β and γ are indicated.

Figure 3 (a) Amino acid sequences of echistatin β and γ and (b) comparison of the primary structures of echistatin variants

SCM-echistatins and their fragments obtained by endoproteinase Arg-C hydrolysis or CNBr cleavage were sequenced by automated Edman degradation. Single-letter amino acid codes are used. An asterisk indicates a non-conserved site.

analysis of the amino acids released by carboxypeptidase Y. The molecular masses of echistatin β and γ calculated from their sequences are 5554 Da and 5433 Da respectively. These are consistent with the results of analysis using electrospray m.s. (model Bio Q triple quadrupole, VG instrument) (results not shown).

Figure 4 Dose-dependent inhibition of platelet aggregation by echistatin α ($\blacktriangle$), β ($\circ$) and γ ($\bullet$)

ADP-induced platelet aggregation was studied with PRP from four species (a) human, (b) rabbit, (c) guinea pig and (d) rat. IC_{50} (nM) is indicated by an arrow. Each data point is the average of those from two experiments.

Species-specificity of the antiplatelet activities of echistatin variants

We have compared the dose-dependent inhibitory effects of the echistatin variants on PRP of various species. IC_{50} values were calculated from the dose-dependence curves (Figure 4). Echistatin β was more potent than α or γ toward human PRP, but for inhibition of rabbit PRP, echistatin γ was the most potent and the IC_{50} values of echistatin β and α were 2–3-fold higher than that of echistatin γ (Figure 4). In general, human and guinea-pig PRP were more sensitive to echistatins than rabbit and rat PRP. Both echistatins α and β showed prominent species specificity, e.g. echistatin β had an IC_{50} for rabbit PRP 14-fold and for rat PRP about 200-fold higher than that for human PRP. In contrast, echistatin γ was less discriminating among platelets of different species tested.

The effects of removal of the C-terminal highly variable residues of echistatin γ were studied using a synthetic truncated form, des-(46–49)-echistatin γ , which could fold properly as shown by its c.d. spectrum and which, like the native form, was a very potent inhibitor of rabbit platelet aggregation [29]. We also found that des-(46–49)-echistatin γ retained the same antiplatelet potency as native echistatin γ toward both human and rabbit PRP.

Identification of disulphide bridges in echistatin γ

There are four disulphide bridges in echistatin α and only two of them (C^2-C^{11} and $C^{20}-C^{39}$) were determined by early n.m.r. study [30]. Here, we used endopeptidase Asp-N and S-DABIA to

determine the other two pairs of disulphides involving Cys residues 7, 8, 32 and 37 in echistatin γ . A polypeptide (R2) containing four disulphides bridges was obtained by proteolysis (Figure 5a). The deduced sequence of R2 is shown in Figure 5(b). This peptide (about 0.5 nmol) was subjected to automated sequencing, and its PTH-amino acid derivatives from steps 5, 7 and 8 were recovered and further reduced by dithiothreitol. Their thiol groups were then modified with S-DABIA and the reddish products were analysed quantitatively by h.p.l.c. with the absorbance monitored at 436 nm. It is expected that if Cys⁷–Cys³² and Cys⁸–Cys³⁷ are present, both steps 7 and 8 of the R2 sequencing would produce comparable PTH-cystine. Alternatively, if it were Cys⁷–Cys³⁷ and Cys⁸–Cys³², step 8 would produce two PTH-cystines and step 7 none. The results (Figures 5c–5f) confirmed the links of Cys⁷ to Cys³² and Cys⁸ to Cys³⁷.

Effect of removal of ARGDDM hexapeptide from echistatin γ

Since the RGD motif of echistatin γ is flanked by Met²² and Met²⁸, the ARGDDM hexapeptide could be removed from echistatin γ by cleavage with 20 mM CNBr in 65% formic acid (25 °C, 20 h). In a control experiment, incubation of echistatin γ in 65% formic acid alone had no adverse effect. The lyophilized and modified protein was purified by h.p.l.c. (Chemcosorb ODS-H) with a linear gradient of 15–30% acetonitrile in 0.07% TFA over 35 min; Des-(23–28)-echistatin γ was eluted at 17 min and native echistatin was eluted at 24 min. Amino acid analysis of des-(23–28)-echistatin γ also confirmed the removal of the ARGDDM loop. The antiplatelet activity of this product was at least 200-fold less than the native protein (80 μ M of des-(23–28)-

Figure 5 Identification of disulphide pairing in echistatin γ

(a) H.p.l.c. purification of fragment R2 from the endoproteinase Asp-N digest of echistatin γ . (b) The deduced amino acid sequences of the two chains in R2 showing two of the disulphide bridges unequivocally identified in echistatin α (solid lines), and those to be confirmed in echistatin γ (dashed lines). (c) H.p.l.c. profile of the synthetic standard S-DAMCAM-(PTH-cysteine). (d–f) H.p.l.c. of the S-DABCAM derivative of the PTH amino acid released at steps 5, 7 and 8 during automated sequencing of R2 respectively. Solvent A was 35 mM acetate buffer, pH 5.0, and solvent B was acetonitrile. Elution was by 20% solvent B isocratic for the first 5 min, 20–40% solvent B over the next 25 min, and then 40% solvent B for 5 min; the flow rate was 1 ml/min.

echistatin γ caused only 20% inhibition). Nevertheless the c.d. spectrum of des-(23–28)-echistatin γ was similar to that of the original protein (not shown). The spectra were characterized by low contents of α -helix and β -sheet and were similar to those reported for echistatin α [30]. The intrinsic fluorescence spectra of des-(23–28)-echistatin γ and the native protein were also similar (not shown).

Displacement of FITC-Rn by echistatin γ and des-(23–28)-echistatin γ

Rn is an RGD-containing peptide and has been demonstrated to be a specific binding ligand for platelet glycoprotein IIb–IIIa [28]. On the removal of Ca^{2+} , glycoproteins IIb and IIIa dissociated and Rn of FITC-Rn failed to bind. The right peak in Figure 6(a) shows increase in fluorescence intensity of human platelets caused by binding of FITC-Rn, in contrast with the EDTA/Tyrod control (the left peak). The mean fluorescence

intensity of the left peak was similar to that of the background (i.e. platelets in the absence of FITC-Rn).

Echistatin γ attenuated the fluorescence of FITC-Rn-treated human platelets in a dose-dependent manner (Figures 6b–6d) indicating that echistatin γ displaced FITC-Rn from the platelet receptors. The IC_{50} of echistatin γ was about 200 nM (Figure 6c), and echistatin γ at 900 nM abolished 89% of FITC-Rn binding (Figure 6d). However, 21 μM des(23–28)-echistatin had no detectable effect on the binding of FITC-Rn (Figure 6e).

Effect of methionine oxidation on the activities and specificities of echistatin β and γ

In the present study, the methionine residues of echistatin β and γ , being several residues away from the RGD motif, were oxidized to methionine sulfoxides with chloramine-T. Figure 7 shows the time-dependent and dose-dependent effect of meth-

Figure 6 Effect of echistatin γ and des(23-28)-echistatin γ on the binding of FITC-Rn to platelets assessed by flow cytometry

Each panel represents the binding profile of 3.2 nM FITC-Rn to human platelets. (a) Distribution of relative fluorescence intensity in the presence of 5 mM EDTA (left peak) and in normal Tyrode solution (right peak), as a result of the association of FITC-Rn with platelet membrane glycoprotein IIb-IIIa. (b-e) Distribution of relative fluorescence intensity in the presence of 18, 180 and 900 nM echistatin γ and 21 μ M des-(23-28)-echistatin γ respectively. The abscissa is a log scale and the ordinate is normal. The values in parentheses are the calculated percentage inhibition of the binding of FITC-Rn (mean $\pm$ S.E.M., $n = 3$). In (a) the mean fluorescence intensities of the negative (left peak) and positive (right peak) control are 1.12 ± 0.03 and 34.0 ± 1.45 respectively.

ionine oxidation on the activity of echistatin toward rabbit PRP. Oxidation was complete in 30 min when 20 μ M echistatin was incubated with 100 μ M chloramine-T. Platelet-aggregation-inhibitory activity of oxidized echistatin β (with methionine sulfoxide at position 28) in rabbit was reduced to about 40-45% of the original, and that of the oxidized echistatin γ (with methionine sulfoxides at 22 and 28) to about 20%. We further compared IC_{50} values of native and oxidized echistatins by assay with both rabbit and human PRP (Figure 8). The potency of [Met²⁸-sulphoxide]echistatin β toward both human and rabbit PRP fell about 2.1-fold. The potency of [Met²², Met²⁸-sulphoxide]echistatin γ decreased 2.5-fold (i.e. 700 nM to 280 nM) toward human PRP but 3.6-fold (i.e. 1300 nM to 360 nM) toward rabbit PRP.

DISCUSSION

The heterogeneity and different anticoagulation activities of *E. carinatus* venoms from different geographic regions have been reported [30]. In the present paper, we have isolated two novel variants of echistatin from the venom of *E. carinatus leakeyi*. The presence of disintegrin isoforms in the venoms of *E. carinatus* subspecies reflects the evolution of these species in different geographic environments.

Echistatin β and γ have 50 and 49 residues respectively including eight cysteines; their protein sequences were respectively 88 and 84% identical with that of echistatin α 1. Echistatin γ has acidic or neutral residues at positions 15, 21 and 22, and

Figure 7 Effect of oxidation of methionine by chloramine-T on the function of echistatin β (○) and γ (●)

(a) Dose-dependence of the effect of chloramine-T treatment for 30 min; (b) time course of the oxidation by 100 μ M chloramine-T and the concomitant reduction in the activities of the echistatins.

thus has a pI value of 4.5, similar to that of carinatin [16]. Echistatin β (pI 9.2) differs from α 1 (pI 8.3) by seven residues. In previous mutagenesis studies of kistrin, a medium-size disintegrin, two mutants F38A and Q60A could not be expressed, presumably because of a folding problem [38]. Residues 38 and 60 of kistrin correspond to Phe¹³ and Lys³⁵ respectively in echistatins. These two residues are conserved in all the echistatins (Figure 3) and many other disintegrins [1-4]. Whether they are important for the folding of small disintegrins remains to be ascertained.

Interestingly, the present study showed that platelets from different species have different sensitivities to the echistatin isoforms. Within human PRP the inhibition follows the order echistatin $\beta > \alpha > \gamma$, and with rabbit PRP the potency order is $\gamma > \beta > \alpha$ (Figure 4). It has been reported [32,33] that some monoclonal antibodies against human platelet glycoprotein IIb-IIIa failed to react with rabbit platelet receptors, and it was recently shown in studies performed with whole blood that rat and hamster platelets were about 100-fold less sensitive than human, monkey and guinea-pig platelets to echistatin α and RGDW [34]. Our study has highlighted the evolution of snake venom disintegrins with respect to platelets of different origin. We have also found that the venom of other Mideast sand vipers including *E. carinatus leucogaster* and *E. carinatus pyramidum* contain echistatin β and γ (but not α) (I.-H. Tsai and Y.-L. Chen, unpublished work). The presence of several disintegrin isoforms in the same venom may have a selective advantage for catching different prey or for defense against various predators. This is also manifested in the simultaneous presence of isoforms of other venom components such as phospholipase A₂ [35].

The sequences of the C-terminal halves of all the echistatin variants are identical except those after residue 46 (Figure 3). We have studied the potencies (or IC_{50}) of the C-terminal truncated synthetic echistatin des-(46-49)-echistatin γ toward both human

Figure 8 Comparison of activities of native and oxidized echistatins toward human and rabbit PRP

Aggregation-inhibition potencies and IC_{50} values (nM) are shown for echistatin β (○) and echistatin γ (●). Solid lines are for data obtained for native echistatins, and dashed lines are for those obtained with chloramine-T-oxidized proteins.

and rabbit PRP. Our results indicate that this truncation did not affect the antiplatelet potency of echistatin γ for either human or rabbit PRP. Therefore we conclude that the difference in species-dependent specificities of echistatins β and γ is not due to variations in their C-terminal regions, but is probably related to differences in residues 15, 21, 22 and 27 (Figure 3), which are close to [30,36] or within the RGD loop. Echistatins α and β apparently have similar species specificity and their residues 15, 21 and 22 are identical (Figure 3).

Previous studies have noted that large hydrophobic side chains in the X position of the RGD sequence are optimal for high-affinity interactions with human platelet glycoprotein IIb-IIIa and possibly other integrins [11,37–39]. Thus the higher activity of echistatin β compared with α and γ in inhibiting aggregation of human platelets (Figure 4a) is at least partly due to the replacement of Asp²⁷ with Asn (Figure 3). However, this cannot be the complete explanation for the less discriminating activity of echistatin γ as echistatin α also has Asp²⁷ but hardly inhibits rabbit platelet aggregation at all (Figure 4b).

Residue Met²⁸, which is conserved in all the echistatin variants, is better than its sulphoxide at inhibiting aggregation of both human and rabbit platelets as suggested by a 2.1-fold inactivation

of echistatin β after the chloramine-T treatment (Figure 8); oxidation of Met²² in addition to Met²⁸ in echistatin γ resulted in a greater reduction (3.6-fold) of its inhibitory activity toward rabbit PRP which is more pronounced than for human PRP (2.5-fold). Thus Met²² of echistatin γ is probably involved in the selectivity in rabbit platelets (Figure 4) but not in human platelets.

On the other hand, Lys²¹ and Arg²² of echistatin β and α probably aid the recognition of human platelets because it was shown by a mutagenesis study of Arg⁴⁶ in kistrin (corresponding to Lys²¹ in echistatin β) that basic charge at this region was important for the binding of kistrin to human glycoprotein IIb-IIIa [40].

There is no direct evidence for the involvement of Glu¹⁵ of echistatin γ in the species-dependent platelet sensitivity. However, of all the disintegrins known, only trigramin α and echistatin γ contain the IEE or LEE sequence at residues 14–16, other disintegrins containing basic residues at the corresponding region. Trigamine α has been shown to have similar activity on rabbit and human platelets (IC_{50} values are 3.1 and 3.4 μ g/ml respectively) (T. F. Huang, unpublished work). In contrast, echistatin α/β and trigramin β/γ have basic substitutions at positions 15 and 22 (echistatin numbering) and apparently inhibit human and guinea-pig PRP more selectively (Figure 4). We therefore conclude that the charge and hydrophobicity of residues 15, 21, 22 and 27 of each echistatin variant determine its species-dependent platelet sensitivity.

Recently, the disulphide links in echistatin $\alpha 1$ were identified by direct chemical analysis [41] and a simulated annealing analysis of the n.m.r. data [42]. Our data suggest that echistatin γ has the same disulphide pattern as echistatin α , i.e. Cys²–Cys¹¹, Cys⁷–Cys³², Cys⁸–Cys³⁷ and Cys²⁰–Cys³⁹. Only Cys²⁰–Cys³⁹ and Cys⁷–Cys³² are highly conserved among all snake venom disintegrins. The disulphide Cys²–Cys¹¹ is probably also important since both Cys² and Cys¹¹ are conserved (although the linkages in which they are involved may vary among different disintegrins) [41]. The importance of Cys⁸–Cys³⁷ of echistatin remains obscure; Cys³⁷ is absent from all medium-size disintegrins.

The great reduction ($\sim 10^2$ – 10^3) in platelet-aggregation-inhibitory potency of des-(23–28)-echistatin γ , i.e. after ARGDDM deletion, suggests the importance of this region for its antiplatelet activity, although other sites in echistatin may also be weakly or indirectly involved in the receptor recognition. For example, the proline-rich C-terminal residues 40–49 in echistatin α have been shown to activate glycoprotein IIb-IIIa for binding to fibrinogen and collagen [43] and have sequences homologous to the human vWF segment 1726–1764 [44]. Moreover, the results of a recent n.m.r. study of other disintegrins suggest that residues corresponding to 42 and 43 of echistatin are probably located close to the RGD motif [36]. However, it required millimolar concentrations of the C-terminal region alone, as suggested by the IC_{50} of the synthetic echistatin α -(40–49)-peptide, for antagonization of GPIIb-IIIa binding to the immobilized echistatin [43]. In the flow-cytometry assay (Figure 6), the lack of activity of 21 μ M des-(23–28)-echistatin in displacing FITC-Rn also suggests that the contribution of non-RGD domains in echistatin to the binding is relatively minor (less than 1% that of the RGD loop). This conclusion is in accord with the results of site-directed mutagenesis studies on a medium-size disintegrin, kistrin [40].

It has been reported that nanomolar concentrations of echistatin α inhibited the attachment of cancer cells to vitronectin and fibronectin [8]. Previous studies [11,37–39] have shown that various RGD-dependent integrins (e.g. $\alpha_v\beta_3$ and $\alpha_5\beta_1$) recognize differences in the conformation and environment of the

RGD motifs of adhesion inhibitors. How the unique N²¹M²² sequence N-terminal to the ARGD tetrapeptide in echistatin γ (corresponding to K²¹R²² in echistatin α) affects its integrin specificity remains to be studied.

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