Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2471–2475, April 1989
Biochemistry

Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: Evidence for a family of platelet-aggregation inhibitors

(Arg-Gly-Asp/fibrinogen receptor/trigramin/echistatin/kistrin)

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Communicated by K. M. Brinkhous, December 22, 1989

ABSTRACT **The purification, complete amino acid sequence, and biological activity are described for several homologous snake venom proteins that are platelet glycoprotein (GP) IIb-IIIa antagonists and potent inhibitors of platelet aggregation. The primary structures of kistrin (from *Agkistrodon rhodostoma*), bitan (from *Bitis arietans*), three isoforms of trigramin (from *Trimeresusus gramineus*), and an isoform of echistatin (from *Echis carinatus*) were determined by automated sequence analysis and fast atom bombardment mass spectrometry analysis. Each of the proteins in this family, which range from 47 to 83 residues, contains an Arg-Gly-Asp amino acid sequence found in protein ligands that binds to GPIIb-IIIa, a high (17 ± 1%) cysteine content conserved in the primary sequence, and a homologous N-terminal region absent only in the echistatin isoforms. Each protein directly inhibits the interaction of purified platelet GPIIb-IIIa to immobilized fibrinogen about 100 times more effectively than does the pentapeptide Gly-Arg-Gly-Asp-Ser; IC_{50} values range from 1.1 to 3.0 nM. The IC_{50} value for the inhibition of platelet aggregation, using human platelet-rich plasma stimulated with ADP, ranges from 110 to 550 nM for the various proteins, about 1000-fold more potent than Gly-Arg-Gly-Asp-Ser. Kistrin binds reversibly to both resting and ADP-activated human platelets with high affinity (K_d = 10.8 nM and 1.7 nM, respectively) and to purified GPIIb-IIIa with a lower affinity (K_d = ≈100 nM). Finally, kistrin injected at 1.0 mg/kg into rabbits reversibly inhibits platelet aggregation *ex vivo* over 30 min without induction of thrombocytopenia. We propose that these proteins are members of a general class of proteins found in the venom of pit vipers that inhibit platelet aggregation by antagonism of the GPIIb-IIIa–fibrinogen interaction and as such serve as potential antithrombotic agents.**

Snake venoms have been widely studied and found to have potent effects on hemostasis through both pro- and anticoagulant mechanisms (1–3). Factors from venoms have been discovered and characterized that affect platelet function by promotion and inhibition of platelet aggregation (4–12). There are numerous independent extracellular sites and intracellular pathways involved in the activation of platelets resulting in aggregation (13), some of which have been targets for therapeutic intervention for thrombotic disease (14). Platelet aggregation is mediated by the interaction of fibrinogen (Fg) with the membrane glycoprotein (GP) IIb-IIIa (15, 16), a member of the integrin family of cell-adhesion receptors (17, 18). The ligand specificity of GPIIb-IIIa is complex; evidence of interaction with other adhesive proteins as well as with peptides derived from putative Arg-Gly-Asp (RGD) or γ-chain recognition sequences of Fg has been reviewed (16). The activation of platelets and GPIIb-IIIa, though poorly understood, is a prerequisite for Fg binding (19). Thus, the binding of Fg to GPIIb-IIIa is a final common event of all activators leading to aggregation and is an excellent target for therapeutic intervention in thrombotic diseases.

Our efforts to isolate and characterize snake venom proteins as specific antagonists to human platelet GPIIb-IIIa that inhibit platelet aggregation have revealed the existence of a family of related proteins. In addition to the purification of isoforms of trigramin and echistatin (7–11), we have isolated two other proteins. The first, which we have named kistrin, is from the venom of the Malayan pit viper *Agkistrodon rhodostoma*, reported to contain a glycosylated 31-kDa platelet aggregation inhibitor (12); the second, which we have named bitan, is from the venom of the puff adder *Bitis arietans*. In this report the complete amino acid sequences for kistrin, bitan, and isoforms of trigramin and echistatin are presented. Data are provided on the ability of the proteins to inhibit Fg binding to purified human platelet GPIIb-IIIa and on the direct binding of kistrin to resting and activated platelets and to purified GPIIb-IIIa. Finally, evidence for *in vivo* activity of kistrin in rabbits is presented, demonstrating the potential use of these proteins as antithrombotic agents. Based on the observed amino acid sequences and activities, we show that these snake venom proteins belong to a homologous family that inhibit platelet aggregation by direct interaction with the platelet integrin receptor GPIIb-IIIa.

MATERIALS AND METHODS

Materials. Lyophilized *A. rhodostoma* and *Trimeresurus gramineus* snake venoms were purchased from Miami Serpenterium Laboratories (Salt Lake City); *B. arietans* and *Echis carinatus* venoms were from Sigma. Human Fg (Kabi-L) was purified by the method of Lipinska *et al.* (20); purity was >94% based on densitometric analysis of silver-stained reduced SDS/polyacrylamide gels and <1% fibronectin was present by a cell attachment assay (21). Platelet GPIIb-IIIa was prepared by the method of Fitzgerald *et al.* (22) and stored frozen (−80°C) in TACTS (20 mM Tris·HCl, pH 7.5/0.02% NaN_3/2 mM $CaCl_2$/0.05% Tween 20/150 mM NaCl) with 0.1% Triton X-100. Densitometric analysis of Coomassie-stained reduced SDS/polyacrylamide gels indicated >95% purity for GPIIb-IIIa. Immunoblot analysis was negative for vitronectin receptor by using specific antibodies (Telios, La Jolla, CA); fibronectin receptor immunoreactivity was detectible, although no protein was detected by silver stain. AP3, a murine monoclonal antibody to human GPIIIa that does not inhibit platelet aggregation (23) was obtained

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Abbreviations: GP, glycoprotein; Fg, fibrinogen; FAB, fast atom bombardment; RGD, Arg-Gly-Asp; GRGDS, Gly-Arg-Gly-Asp-Ser; F_3CCOOH, trifluoroacetic acid; MS, mass spectrometry; BSA, bovine serum albumin; PRP, platelet-rich plasma.

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from P. Newman (Blood Center at Southeastern Wisconsin, Milwaukee). ^{125}I-labeled kistrin was prepared using Iodobeads (Pierce) followed by gel filtration on Sephadex G-25. The peptide Gly-Arg-Gly-Asp-Ser (GRGDS) was synthesized using t-Boc chemistry (24) and purified on a reverse-phase (rp) C_{18} column using a 0.1% trifluoroacetic acid (F_3CCOOH)/acetonitrile gradient; amino acid and mass spectral analyses confirmed the composition. The thromboxane A_2 mimetic U-46619 was a gift from Upjohn.

Purification of Snake Venom Proteins. Lyophilized venom (50 mg in 1.5 ml of H_2O) was loaded onto a Sephadex G-50 (fine) column (100 × 1 cm^2) equilibrated in 50 mM Tris·HCl (pH 7.5) containing 0.5 mM $CaCl_2$ and 0.02% NaN_3. The column flow rate was 0.1 ml/min; 1.75-ml fractions were collected. Fractions were assayed using the Fg/GPIIb-IIIa ELISA. Active fractions were pooled and purified by HPLC using a 5-μm, 250 × 4.6 mm Vydac C_{18} column (218TP-54) and a 0.1% F_3CCOOH/acetonitrile gradient at 0.5–1.0% per min from 5–10% to 30–70% (vol/vol) acetonitrile in 0.1% F_3CCOOH; proteins were monitored at A_{214}.

Amino Acid Analysis. Samples were cleaved by constant boiling 6 M HCl vapor in the Millipore Picotag system for 20 hr at 110°C. The hydrosylates were dried and analyzed on a Beckman model 6300 amino acid analyzer.

Reduction and Carboxymethylation. Each purified snake venom protein (≈10 μg) was dried *in vacuo* and solubilized in 100 μl of 6 M guanidine hydrochloride/10 mM Tris, pH 8.3/1 mM EDTA/2 mM dithiothreitol for 1 hr at 37°C. After addition of iodoacetic acid (6 mM) for 1 hr at 37°C in the dark, the carboxymethylated sample was isolated by HPLC as described above. The reduced and carboxymethylated proteins were eluted about 10 min later than the native proteins.

Fast Atom Bombardment-Mass Spectrometry (FAB-MS). Samples (≈50 pmol) were lyophilized, redissolved in 2–3 μl of 70% (vol/vol) formic acid, dried onto the probe tip, and resuspended in 1.4 μl of thioglycerol or *m*-nitrobenzyl alcohol/70% formic acid, 50:50 (vol/vol). FAB-MS data were obtained on a JEOL model HX110/110 tandem mass spectrometer operated in the two-sector mode.

Sequence Analysis. Native proteins (0.5 nmol) were loaded onto a model 470A Applied Biosystems gas-phase sequencer equipped with a 120A phenylthiohydantoin amino acid analyzer. Prior to analysis, echistatin-α2 (1 nmol) was incubated in 0.1 ml of 50 mM sodium phosphate, pH 7.3/1 mM EDTA/10 mM 2-mercaptoethanol, containing 0.5 mg of pyroglutamate aminopeptidase (Boehringer) for 3 hr at 50°C. Phenylthiohydantoin amino acids were integrated with a Nelson Analytical model 3000 data system; data analysis was performed on a VAX 6850 (Digital Equipment) (25).

Each purified, reduced, and carboxymethylated lyophilized protein (10 μg) was digested with 1 μg of endoproteinase Lys-C (Boehringer) in 100 μl of 10 mM Tris, pH 8.5/1 mM EDTA/10% acetonitrile for 6 hr at 37°C. For CNBr digests, 1 nmol of reduced and carboxymethylated, HPLC-purified, lyophilized protein was solubilized in 100 μl of 80% formic acid containing 0.1 mg of CNBr in the dark for 20 hr at 25°C and diluted 1:2 with water. Lys-C and CNBr digests were separated by reversed-phase HPLC as described above. Isolated peptides were sequenced and analyzed by FAB-MS.

Fg/GPIIb-IIIa ELISA. The Fg/GPIIb-IIIa ELISA was performed by a modification of the method of Nachman and Leung (26). Microtiter plates were coated with purified human Fg (10 μg/ml) and then incubated with TACTS/0.5% bovine serum albumin (BSA). After washing with phosphate-buffered saline (PBS) containing 0.01% Tween 20, samples to be evaluated were added, and then purified GPIIb-IIIa (40 μg/ml) in TACTS/0.5% BSA was added. After a 1-hr incubation, the plate was washed and monoclonal antibody AP3 (1 μg/ml) was added; AP3 (25 μg/ml) did not displace bound GPIIb-IIIa. After a 1-hr incubation and another wash, goat anti-mouse IgG conjugated to horseradish peroxidase (Tago) was added. A final wash was performed and developing reagent buffer (0.67 mg *o*-phenylenediamine dihydrochloride per ml/0.012% H_2O_2/22 mM sodium citrate/50 mM sodium phosphate, pH 5.0) was added; plates were incubated until color developed. The reaction was stopped with 2 M H_2SO_4 and A_{492} was recorded.

Platelet Aggregation Assay. Platelet aggregation assays were performed in human platelet-rich plasma (PRP) as follows: 50 ml of whole human blood (9 parts) was drawn on 3.8% (wt/vol) sodium citrate (1 part) from donors who had not taken any aspirin or related medication for at least 2 weeks. Blood was centrifuged (160 × *g* for 10 min) at 22°C and allowed to stand for 5 min, and the PRP was decanted. Platelet-poor plasma (PPP) was prepared from the remaining blood by centrifugation (2000 × *g* for 25 min) at 22°C. PRP platelet count was measured on a Baker model 9000 hematology analyzer and diluted to 300,000 platelets per μl with PPP.

PRP (225 μl) plus 25 μl of either a venom protein in PBS or PBS alone was incubated for 5 min in a Chrono-log Aggregometer at 25°C. An aggregating agent [collagen (1 μg/ml), U46619 (100 ng/ml), or ADP (8 μM)] was added and the light transmittance was recorded; inhibition was measured at the maximum aggregation response.

Binding of ^{125}I-Labeled Kistrin to Washed Human Platelets. Washed platelets were prepared from PRP incubated with prostaglandin I_2 (at 300 ng/ml; Sigma) for 10 min followed by apyrase (5 units/ml, grade III; Sigma) for an additional 10 min (27, 28). The platelet pellet was obtained by consecutive centrifugation (1600 × *g* for 7 min at 25°C) and resuspension first in 10 ml of Tyrode's buffer with 5.5 mM glucose and 2% BSA (Tyrode's/BSA) containing prostaglandin I_2 (300 ng/ml) and apyrase (1 unit/ml), then in 10 ml of Tyrode's/BSA with apyrase (0.2 unit/ml), and finally in Tyrode's/BSA. The platelets, diluted to ≈50,000 platelets per μl, were incubated for 2 hr before use.

Washed platelets (200 μl at 1.1–1.8 × 10^7 platelets per ml) were incubated with ^{125}I-labeled kistrin (11.6 μCi/μg; 2.5–1000 nM; 1 Ci = 37 GBq) and 2 mM $CaCl_2$ in Tyrode's/BSA in the absence or presence of 20 μM ADP for 60 min at 25°C. Aliquots (150 μl) were layered on 750 μl of Tyrode's/BSA/20% (wt/vol) sucrose in Eppendorf tubes and centrifuged (14,000 × *g* for 6 min). The liquid was aspirated and radioactivity in the cut tips containing the platelet pellet was measured in an Iso-Data series 100 γ counter.

***In Vivo* Rabbit Studies.** Male New Zealand White rabbits were anesthetized with a mixture of 7.5 mg of fluanisone and 0.24 mg of fentanyl, and blood samples were drawn onto 3.8% sodium citrate (9:1) before and at 5, 10, 15, 30, 45, and 60 min after dosing. PRP was prepared by rapid centrifugation (2 sec) at 14,000 × *g* of whole blood; *in vitro* aggregation responses to collagen (10 μg/ml; Chrono-Par type I from equine tendon) were determined at the maximum aggregation response and platelet counts were measured in whole blood and PRP as described above.

RESULTS

Purification. Snake venom proteins were purified to apparent homogeneity from the venoms of *A. rhodostoma, B. arietans, T. gramineus*, and *E. carinatus* by gel-filtration chromatography (Sephadex G-50) followed by rpHPLC (C_{18}). To identify and purify specific GPIIb-IIIa antagonists, a modified Fg/GPIIb-IIIa ELISA (26) was developed that measures the inhibition of the binding of soluble purified human platelet GPIIb-IIIa to immobilized Fg. The activity of the purified proteins was subsequently characterized by measuring *in vitro* inhibition of platelet aggregation. Three peaks of activity from the venom of *A. rhodostoma* were detected after rp chromatography; the major protein peak represented 1.5% of the crude venom (dry weight). One peak

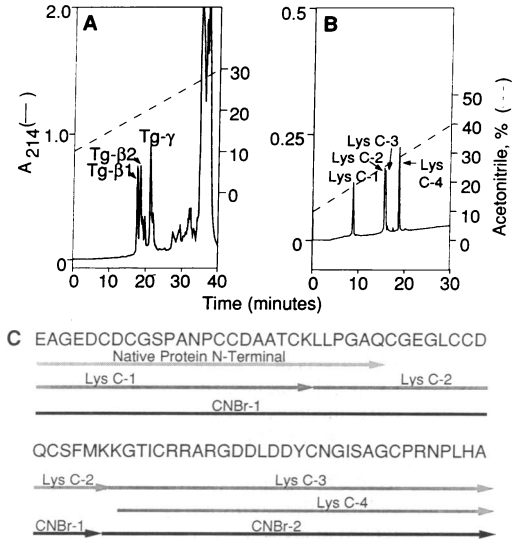

FIG. 1. Purification and sequence determination for trigramin-γ. (*A*) rpHPLC purification of trigramin isoforms. Active fractions from a Sephadex G-50 gel-filtration column were further purified by rpHPLC using an acetonitrile gradient containing 0.1% F_3CCOOH. The three major active fractions (Tg-β1, Tg-β2, and Tg-γ) were detected. (*B*) Separation by rpHPLC of peptides generated by Lys-C proteolysis of reduced and carboxymethylated Tg-γ. (*C*) Amino acid sequence analysis of native trigramin-γ and the Lys-C and CNBr fragments used to align the sequence. Native protein and Lys-C peptides were sequenced by Edman degradation; the arrows indicate the length of sequence obtained and are in agreement with the mass for each of the fragments as determined by FAB-MS (data not shown). Partial digestion at Lys-43 in the sequence gave rise to a mixture of Lys-C-3 [Lys-Gly-Thr-Ile-Cys-Arg- (KGTICR-)] and Lys-C-4 [Gly-Thr-Ile-Cys-Arg- (GTICR-)], indicative of adjacent lysines in this region. This overlap was confirmed from the sequence of CNBr-2, produced by CNBr treatment of Tg-γ. The single-letter amino acid code is used.

of activity was observed for *E. carinatus* that was isolated as 0.4% of the crude venom. There were four major and several minor peaks of activity observed from rpHPLC purification of the venom from *B. arietans*; only one of the major proteins, bitan-α, representing 1% of the crude venom, was further characterized. In contrast to earlier observations (7–9), we found three major and three minor active protein peaks by rpHPLC of *T. gramineus* venom (Fig. 1*A*). The three major activity peaks, designated Tg-β1, Tg-β2, and Tg-γ, were isolated as 0.18%, 0.17%, and 0.4% of the crude venom, respectively. Each protein was purified to homogeneity by rechromatography on rpHPLC and characterized.

Amino Acid Sequence, Composition, and FAB-MS Analysis. The amino acid sequences of kistrin, bitan, and the isoforms of trigramin and echistatin were determined by Edman degradation of the intact reduced and carboxymethylated proteins and from peptides derived by Lys-C proteolytic digestion; an HPLC chromatogram for the Lys-C digest of trigramin-γ is shown in Fig. 1*B*. The N terminus of echistatin-α2 was blocked; sequence was obtained only after treatment with pyroglutamylaminopeptidase. In general, the order of the first two Lys-C peptides was established by N-terminal sequence analysis of the intact protein. Partial digests with Lys-C and digests with CNBr were performed subsequently to provide the overlaps necessary to complete the amino acid sequence; peptide fragments used to determine the sequence of trigramin-γ are shown in Fig. 1*C*. Amino acid composition for each protein agreed with that predicted from the protein sequence. The complete sequences for all of the proteins are shown in Fig. 2.

FAB-MS analysis of isolated peptide fragments and native molecules was used to corroborate the sequence analysis and to verify that complete sequences had been obtained. An excellent correlation was found between the observed and calculated molecular masses of both the native proteins (Table 1) and the peptide fragments (data not shown). The mass spectral data for the native proteins are consistent with all the cysteines forming disulfide bonds. In addition, alkylation of the native proteins by iodoacetic acid was observed only after reduction with dithiothreitol.

Native echistatin-α2 was resistant to CNBr cleavage even though amino acid sequence analysis (Fig. 2) and composition (data not shown) indicated one methionine; this is consistent with oxidation of the methionine (29). The presence of Met-28 sulfoxide was confirmed by mass spectral data (Table 1). This sequence differs from echistatin-α1 (10) in that it lacks the Ala-Thr residues at the C terminus, contains an N-terminal pyroglutamyl group (likely derived from cyclization of glutamine, which is conserved in all of the other proteins at this position), and contains an oxidized methionine. A minor peak in the kistrin purification showed an M+16 peak in the mass spectrum for both the native protein and the Lys-C methionine-containing peptide; the masses of the other Lys-C fragments were identical to those from kistrin. Native kistrin oxidized with 0.5% H_2O_2 in 0.1% F_3CCOOH produced the identical species, most likely the Met-52 sulfoxide, and is designated $kistrin_{ox}$. The precise *in vivo* native protein structures are unknown due to the potential for chemical oxidation or hydrolysis during both the venom extraction and protein isolation procedures.

Inhibition of GPIIb-IIIa–Fg Interaction and Human Platelet Aggregation. The determination of the specific activity (IC_{50}) of each of the snake venom proteins and the peptide GRGDS, a member of RGD-containing peptides that bind to GPIIb-IIIa (30), was measured by their ability to inhibit the binding of purified human platelet GPIIb-IIIa to immobilized Fg, by using the Fg/GPIIb-IIIa ELISA (Table 2). Reduced and carboxymethylated kistrin was inactive in the ELISA, implying that the tertiary structure of the molecule is critical for activity. This is consistent with the loss of platelet aggregation inhibition observed previously for reduced and alkylated trigramin-α and echistatin-α1 (7, 10). A dose-dependent inhibition of platelet aggregation in ADP-stimulated human PRP was observed for each of the snake venom proteins that

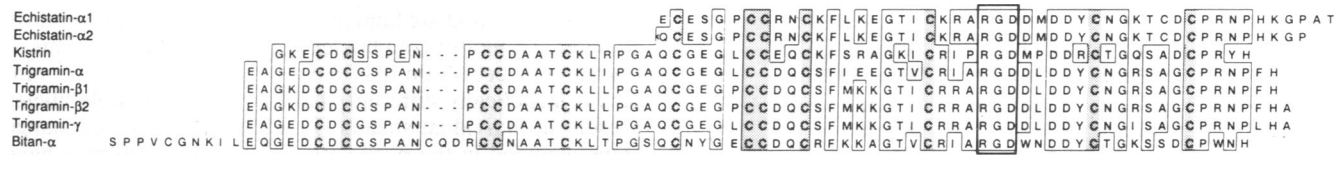

FIG. 2. Sequence alignment of the snake venom GPIIb-IIIa antagonists. The sequences of echistatin-α1 [49], -α2 [47], kistrin [68], trigramin-α [72], -β1 [72], -β2 [73], and -γ [73], and bitan-α [83] are aligned (number of residues are in brackets). The sequences of echistatin-α1 and trigramin-α have been reported (8, 10). Residues that are conserved in >50% of the sequences are boxed; conserved cysteine residues are shaded; and the RGD region common to each of the inhibitors is in a bold box. The <Q at the N terminus of echistatin-α2 refers to a pyroglutamyl residue. The methionine at position 28 in echistatin-α2 is oxidized to the sulfoxide; the methionine at position 52 of kistrin can be readily oxidized to the sulfoxide by H_2O_2 to produce $kistrin_{ox}$. The single-letter amino acid code is used.

Table 1. FAB-MS spectrometry of snake venom protein GPIIb-IIIa antagonists

Antagonist	Molecular mass, amu	
	Observed*	Calculated†
Kistrin	7318 ± 2	7318.30
$Kistrin_{ox}$	7334 ± 2	7334.30
Bitan-α	8987 ± 4	8989.98
Trigramin-β1	7551 ± 2	7550.49
Trigramin-β2	7623 ± 2	7621.57
Trigramin-γ	7563 ± 2	7561.51
Echistatin-α2	5243 ± 2	5242.95‡

*Data reported are corrected for the ionized M+1 (H^+) or M+23 (Na^+) peak that is observed.
†Molecular mass data were calculated from the sequence of the native proteins, assuming all cysteines form disulfide bonds.
‡Calculated mass assumes an N-terminal pyroglutamate and the methionine oxidized to the sulfoxide.

was ≈1000-fold more potent than for GRGDS (Table 2). The platelet aggregation IC_{50} values for kistrin are independent of the platelet activator, consistent with GPIIb-IIIa as a final common mediator of platelet aggregation (15, 16). The 100- to 1000-fold greater potency of the inhibitors in the ELISA relative to the more biologically relevant platelet aggregation assay is noteworthy. This probably results from the combined effects of Fg immobilization, low GPIIb-IIIa concentration, and differences in the affinity of purified GPIIb-IIIa relative to that in the intact platelet.

Binding of Kistrin to Human Platelets and GPIIb-IIIa. Scatchard analysis of the binding of ^{125}I-labeled kistrin to unactivated and ADP-activated human washed platelets yielded K_d values of 10.8 ± 1.8 nM (72,000 sites) and 1.7 ± 0.2 nM (79,000 sites), respectively (Fig. 3). Activation of platelets with ADP resulted in increased binding affinity without a significant change in the number of binding sites on the platelet and was consistent with the number of sites determined by binding studies with Fg. Kistrin (100 nM) completely inhibited ^{125}I-labeled Fg binding to washed platelets; an IC_{50} of ≤10 nM was observed (M.A.N., unpublished data). In contrast, ^{125}I-labeled Fg does not bind to unactivated platelets (32, 33) and binds with low affinity to ADP-activated platelets; a K_d value of ≈100 nM was measured by competition or saturation binding consistent with reported data (32, 33). The binding of ^{125}I-labeled kistrin to resting and ADP-stimulated platelets was reversible, based on the addition of a 100-fold excess of unlabeled kistrin, after a 60-min

Table 2. GPIIb-IIIa antagonist activity summary

Compound	IC_{50}, nM	
	Fg/GPIIb-IIIa solid-phase ELISA	Human platelet aggregation assay
Kistrin	2.7 ± 1.4 (5)	128 ± 35 (4) 135 ± 15 (2) [U46619] 105 ± 25 (2) [collagen]
$Kistrin_{ox}$	2.4 ± 0.7 (3)	138 ± 40 (2)
$Kistrin_{red/cm}$	>1500 (1)	ND
Bitan-α	1.8 ± 0.4 (3)	108 ± 2 (2)
Trigramin-β1	3.0 ± 1.2 (4)	300 ± 80 (3)
Trigramin-β2	2.3 ± 0.1 (2)	170 (1)
Trigramin-γ	2.2 ± 0.2 (2)	240 (1)
Echistatin-α2	2.7 ± 0.5 (3)	555 ± 55 (2)
GRGDS	205 ± 70 (3)	225,000 ± 70,000 (3)

IC_{50} value is the concentration necessary to inhibit total platelet aggregation in PRP to 50% of the control aggregation. Values are reported for ADP activation unless noted otherwise. Numbers in parentheses refer to the number of independent determinations. ND, not done.

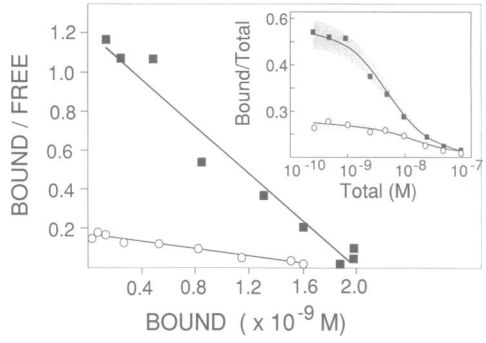

FIG. 3. Scatchard analysis of ^{125}I-labeled kistrin saturation binding to unactivated and ADP-activated washed human platelets. Washed platelets were incubated with ^{125}I-labeled kistrin in the absence (○) or presence (■) of 20 μM ADP for 60 min at 25°C. All samples were done in triplicate. A ^{125}I-labeled Fg binding control (− ADP, 7495 cpm; + ADP, 20,370 cpm) showed ADP stimulation of the platelets. Data derived from the saturation binding curve (*Inset*) were analyzed using Scatchard analysis (31). The confidence interval (±2 SD) is shown in the shaded area (*Inset*).

preincubation at 22°C. This is in contrast to Fg, which has time-dependent irreversible binding (34), and to trigramin-α, which has been reported to be irreversibly bound after 5 min (7). The apparent dissociation rate of kistrin from activated platelets was ≈2.5-fold slower than from unactivated platelets with 50% dissociation at 25 and 10 min, respectively (M.A.N., unpublished data). Kistrin also binds to purified GPIIb-IIIa in solution, but with a K_d value of ≈100 nM, which is substantially weaker than that observed for the intact platelets. This may be due to conformational differences of the purified receptor or the use of detergent (0.1% Triton X-100) in the assay. These results suggest that members of this family of snake venom proteins act as antagonists by direct binding to GPIIb-IIIa.

***In Vitro* and *in Vivo* Rabbit Platelet Aggregation Studies.** To further investigate the biological activity of this protein family, we studied the *in vitro* and *in vivo* effects of kistrin on rabbit platelets. *In vitro* platelet aggregation in rabbit PRP induced by collagen (10 μg/ml) was inhibited to 50% of control values by 1.2 ± 0.1 μM kistrin. For *in vivo* evaluation, kistrin was administered as a single bolus intravenous injection (1.0 mg/kg); platelet aggregation was then measured *ex vivo* at various times (Fig. 4). Collagen-induced aggregation was inhibited by ≈70% after 5 min and returned to control values within 30 min; the platelet count remained constant throughout the experiment. This transient effect, consistent with reversible binding of kistrin to human GPIIb-IIIa (*vide supra*), may result from rapid clearance or proteolytic degradation of the active species from the circulation. Plasma kistrin levels as measured by immunoassay paralleled the biological effects (S.B., unpublished data).

DISCUSSION

We have isolated a family of unique proteins from the venoms of pit vipers from the genera *Trimeresurus, Bitis, Agkistrodon*, and *Echis* that are human platelet GPIIb-IIIa antagonists and potent inhibitors of platelet aggregation. The isoforms of trigramin or echistatin may have resulted from pooled venoms of genetically different snakes or from multiple alleles present in individual snakes. In addition, the ion-exchange steps included in the purification schemes (7–10) may have resulted in the removal of the trigramin and echistatin isoforms identified in this study. Additional snake venoms containing GPIIb-IIIa antagonist activity have been found (M.S.D., unpublished data) and likely contain proteins in this family. Species with the highest levels of activity in the crude venom are all pit vipers including the genera *Bothrops* and

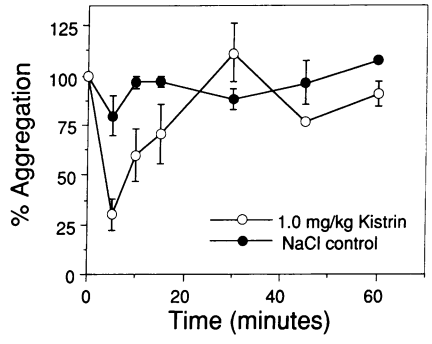

FIG. 4. Inhibition of platelet aggregation (*ex vivo*) after intravenous administration of kistrin to rabbits. Kistrin (1.0 mg/kg) or sterile NaCl was administered to rabbits as a 5-ml i.v. bolus ($n = 5$ for both groups). Results are expressed as percent aggregation compared to the preinjection sample. Data are mean ± SEM. Platelet counts were constant. The apparent inhibition observed for the NaCl control at 5 min was due to the response of one animal; the average of the other four animals was 98.1% aggregation.

Crotalus; however, not all pit vipers were found to possess activity. Although the natural role of these proteins is unknown, they may promote the toxic effects of the snake venom on its prey through an antithrombotic mechanism.

Within the family of snake venom platelet aggregation inhibitors sequenced, there are strong sequence homologies (Fig. 2) and essentially identical GPIIb-IIIa antagonist activities (Table 2). These proteins contain from 47 to 83 residues and four to seven disulfide bonds. The highly conserved and completely oxidized cysteines suggest that the disulfide linkages are similar. However, for echistatin, at least one of the disulfides must be different. Each of the proteins contains the RGD recognition sequence, common to many adhesion proteins (15–18, 35) near the C terminus, that likely accounts for much of the binding interaction with GPIIb-IIIa. However, the snake venom proteins are ≈100-fold and 1000-fold more potent than the peptide GRGDS for inhibition of GPIIb-IIIa binding to Fg or platelet aggregation, respectively. This indicates that other determinants within the protein are important for binding and/or that the RGD is conformationally restrained in the protein in a manner that enhances binding. Although not observed in the Fg/GPIIb-IIIa ELISA, there is a correlation of lower IC_{50} values in the platelet aggregation assay with proteins having a longer N terminus, suggesting that this region may play some role in binding.

The binding and platelet inhibitory effect of kistrin is rapidly and fully reversible, in contrast to other agents, such as aspirin (14), inhibitory monoclonal antibodies to GPIIb-IIIa (36), or, surprisingly, trigramin-α (7). This may offer a marked advantage for the careful control of therapy and quick reversal of any undesired side effects. The interactions of the proteins in the snake venom family to other integrins that bind RGD peptides, such as the vitronectin $\alpha_V\beta_3$ or fibronectin $\alpha_5\beta_1$ receptors (35, 37, 38), is of particular significance since the actual location of these receptors relative to the biodistribution of therapeutic agents may determine their ultimate effectiveness. A report has implied (39) that trigramin-α may block vitronectin or fibronectin adhesion to melanoma or fibroblast cell lines, respectively.

The study of this protein family from both a structural as well as a genetic perspective will be of interest to protein chemists and evolutionary biologists. In addition, they will be of great importance to scientists studying integrin receptors, in particular, GPIIb-IIIa and its role in platelet aggregation. Finally, the therapeutic potential of these proteins has promise in the treatment of thrombotic disease.

We thank Craig Muir for Fg/GPIIb-IIIa ELISA data; Sherron Bullens for platelet aggregation data; Jim Bourell for mass spectral data; Byron Nevins for amino acid analyses; John Burnier for peptide synthesis; Jim Wells, Paul Carter, and Sarah Bodary for a critical reading of the manuscript; and Mike Ross for his support.

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