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Agkistrodon piscivorus piscivorus platelet aggregation inhibitor: A potent inhibitor of platelet activation

(platelet aggregation/platelet secretion/Arg-Gly-Asp/glycoprotein IIb/IIIa/trigramin)

BETTY H. CHAO*, JOSEPH A. JAKUBOWSKI†‡, BRIAN SAVAGE§, E. PING CHOW*, ULLA M. MARZEC§, LAURENCE A. HARKER§, AND JOHN M. MARAGANORE*

*Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142; †Department of Hemostasis Research, Veterans Administration Medical Center, Boston, MA 02130; and §Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, CA 92037

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ABSTRACT **Applaggin (*Agkistrodon piscivorus piscivorus* platelet aggregation inhibitor) is a potent inhibitor of platelet activation. The protein is isolated from the venom of the North American water moccasin snake in three steps, including gel filtration, cation exchange, and reverse-phase HPLC procedures. The purified protein migrates as a 17,700-Da polypeptide by SDS/PAGE under nonreducing conditions and as a 9800-Da peptide in the presence of thiol. The behavior of applaggin on SDS/PAGE would indicate that the protein is a disulfide-linked dimer. Applaggin has been completely sequenced by Edman degradation and consists of 71 amino acids. The sequence is rich in cysteine and contains Arg-Gly-Asp at residues 50–52. Applaggin blocks platelet aggregation induced by ADP, collagen, thrombin, or arachidonic acid with IC_{50} values ranging from 12 to 128 nM (0.2–2.3 μg/ml) depending on the agonist and its concentration. This inhibition is found to correlate with inhibition of thromboxane A_2 generation and of dense granule release of serotonin. Inhibition by applaggin of serotonin release induced by ADP, γ-thrombin, and collagen was monitored in plasma under stirred conditions with [^{3}H]serotonin-loaded platelets, and IC_{50} values for inhibition are found to range from <10 to 145 nM. At saturating concentrations, ^{125}I-labeled applaggin (^{125}I-applaggin) binds to 28,500 sites per unstimulated, washed platelet with a K_d of 1.22 × 10^{-7} M. Binding of ^{125}I-applaggin to platelets is inhibited by the synthetic undecapeptide Arg_8-Gly-Asp-Val at 200 μM.**

Platelet aggregation and release reactions are essential for effective primary hemostasis. Perturbations in mechanisms of platelet recruitment may also affect arterial thrombus formation. Thus, developing inhibitors of platelet aggregation and release has been central to intervention strategies for arterial thromboembolic disease. One approach to the interruption of platelet-rich arterial thrombi has used agents that block the function of platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa). GPIIb and GPIIIa associate via extracellular Ca^{2+} bridges (1) to form a heterodimeric complex of 265 kDa in stimulated and unstimulated platelets (2, 3). Several lines of evidence demonstrate that GPIIb/IIIa is the receptor for fibrinogen (for review, see ref. 4). As a consequence of platelet activation, fibrinogen, which contains four to six separate sites for GPIIb/IIIa binding (5, 6), crosslinks platelets mediating the aggregation response. In addition, platelet production of thromboxane A_2 (TXA_2), and release of α-granule and dense-granule constituents accompanies aggregation and further enhances platelet recruitment. Peptides (7) and monoclonal antibodies (3, 8–10) that bind to GPIIb/IIIa and thereby block platelet aggregation have been reported not to prevent platelet secretion. Similarly, patients with Glanzmann thrombasthenia, a congenital deficiency of GPIIb/IIIa (11), have platelets with impaired aggregation but normal secretion responses (12).

The antithrombotic efficacy of agents that interrupt platelet aggregation via GPIIb/IIIa binding has been demonstrated. Peptides derived from the fibrinogen sequence, such as Arg-Gly-Asp-Ser, block platelet aggregation in plasma at 10^{-4} M concentrations (7). Recently, high concentrations of Arg-Gly-Asp-Val have been shown to interrupt platelet deposition on a collagen-coated device placed in an exteriorized arteriovenous shunt in baboons (13). In a related experimental model, substantially lower concentrations of anti-GPIIb/IIIa monoclonal antibodies AP-2 and LJ-CP8 are required to interrupt the formation of platelet thrombus (14). The anti-GPIIb/IIIa monoclonal antibody 7E3 has been demonstrated to be a potent inhibitor of *in vivo* platelet aggregation (15) and of thrombosis (16) and rethrombosis following thrombolytic reperfusion (17) in experimental animals. Trigramin, an Arg-Gly-Asp-containing polypeptide from the venom of *Trimeresurus gramineus*, blocks *in vitro* platelet aggregation via GPIIb/IIIa binding (K_d = 10^{-7} M) and has been proposed as an alternative inhibitor to anti-GPIIb/IIIa antibodies (18).

We report here identification, purification, and characterization of applaggin (*Agkistrodon piscivorus piscivorus* platelet aggregation inhibitor) from the venom of the North American water moccasin snake. Like anti-GPIIb/IIIa antibodies and trigramin, applaggin blocks platelet aggregation at nanomolar concentrations. However, unlike trigramin, we show that applaggin exists as a disulfide-linked homodimer. Furthermore, applaggin is demonstrated to block both platelet aggregation and secretion reactions.

EXPERIMENTAL PROCEDURES

Materials. Lyophilized venom from *A. p. piscivorus* was obtained from the Miami Serpentarium Laboratories (Salt Lake City, UT). Sephadex G-50-50 and S-Sepharose (fast flow) were obtained from Sigma. Collagen used in TXA_2 generation studies was from Hormon-Chemie (Munich). Otherwise, collagen was purchased from Biodata (Hatboro, PA) as was ADP and arachidonic acid. Human thrombins were a gift of John W. Fenton II (New York State Department of Health, Albany). [^{3}H]Serotonin creatinine sulfate was pur-

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Abbreviations: applaggin, *Agkistrodon piscivorus piscivorus* platelet aggregation inhibitor; TFA, trifluoroacetic acid; PRP, platelet-rich plasma; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; GPIIb/IIIa, glycoprotein IIb/IIIa; RCM-applaggin, reduced *S*-carboxymethylcysteinyl applaggin; RPE-applaggin, reduced *S*-pyridylethyl applaggin; C-RCM-applaggin, RCM-applaggin cleaved with α-chymotrypsin; T-RCM-applaggin, RCM-applaggin cleaved with trypsin.

‡Current address: Department of Cardiovascular Pharmacology, Lilly Research Laboratories, Indianapolis, IN 46285.

chased from Amersham. Solvents and reagents used in structural analyses were of the highest grade available and were purchased from Pierce, Baker, Applied Biosystems, and Beckman.

Purification of Applaggin. The first step in the isolation of applaggin from *A. p. piscivorus* venom (0.5 g per 10 ml) used gel-filtration chromatography with a modification of the method of Ouyang *et al.* (19). The solubilized venom was centrifuged at 3000 × *g* for 10 min and the clarified solution was applied to a column (2.5 × 90 cm) of Sephadex G-50-50 at room temperature. Fractions of 2.0 ml were collected and measured for absorbance at 280 nm. Aliquots of fractions were analyzed for platelet aggregation inhibitory activity using fresh, human platelet-rich plasma (PRP) stimulated with collagen (1.0 μg/ml). Fractions containing anti-platelet activity were pooled and applied to a column (75 ml) of S-Sepharose (fast flow) equilibrated and developed at room temperature in 25 mM Tris·HCl (pH 7.5). This cation-exchange step allowed recovery of applaggin in the column effluent and adsorption of contaminating proteins. Aliquots (0.5 ml) of the S-Sepharose pool were applied to reverse-phase HPLC using a Vydac C4 column (0.46 × 25 cm) and an Applied Biosystems 150 A liquid chromatographic system. The column was equilibrated in 0.1% trifluoroacetic acid (TFA) water and developed with a linear gradient of increasing acetonitrile concentration from 0% to 30% in the same TFA-containing solvent over 60 min at a flow rate of 1.0 ml/min. The column eluate was monitored for absorbance at 214 and 280 nm. Fractions of 0.5 ml were collected, dried in a Speed-Vac instrument (Savant), redissolved in 0.1 ml of water, and tested for anti-platelet activity as described above.

Structural Studies on Applaggin. SDS/PAGE analysis of applaggin under reducing and nonreducing conditions was performed on 19% polyacrylamide gels stained with Coomassie blue to visualize protein. Amino acid analysis of applaggin and reduced *S*-carboxymethylcysteinyl applaggin (RCM-applaggin) was performed by acid hydrolysis with 6 M HCl at 110°C for 24 hr *in vacuo*. Hydrolyzates were analyzed by ion-exchange chromatography and post-column ninhydrin derivatization with a Beckman 6300 amino acid analyzer. Automated Edman degradation of intact and *S*-pyridylethyl applaggin and of peptides derived therefrom was performed on an Applied Biosystems 470A gas-phase sequencer equipped with a model 900A data system. Phenylthiohydantoin (PTH)-derivatized amino acids were analyzed on-line with an Applied Biosystems 120A PTH analyzer and a PTH-C18 column (2.1 × 220 mm).

Determination of the complete amino acid sequence of applaggin involved NH_2-terminal sequence analysis of intact and reduced *S*-pyridylethyl applaggin (RPE-applaggin), proteolytic degradation of RCM-applaggin with α-chymotrypsin (C-RCM-applaggin) and trypsin (T-RCM-applaggin), isolation of C-RCM-applaggin and T-RCM-applaggin fragments by reverse-phase HPLC, and automated Edman degradation of these peptides. Applaggin was denatured in a buffer containing 0.1 M Tris·HCl (pH 8.0), 6 M guanidinium chloride, and 1 mM EDTA. RPE-applaggin was prepared with 0.2 mg of protein in 0.5 ml of denaturing buffer by the method of Friedman *et al.* (20). RCM-applaggin was prepared with 0.2 mg of protein in 0.5 ml of denaturing buffer by the method of Hirs (21). Chymotryptic digests were performed by treatment of 0.2 mg of RCM-applaggin with 2% (wt/wt) α-chymotrypsin for 4 hr at 37°C. The C-RCM-applaggin fragments were purified by reverse-phase HPLC on a Vydac octadecylsilyl column (0.46 × 25 cm) equilibrated in 0.1% TFA/water and developed with an increasing concentration of acetonitrile in the same TFA-containing solvent. The gradient elution used two steps from 0% to 28% acetonitrile over 60 min and 28% to 70% acetonitrile over 5 min at a flow rate of 1.0 ml/min. The effluent stream was monitored at both 214 and 280 nm for absorbance. Fractions were collected manually based on peak detection, dried under vacuum, and subjected to automated Edman degradation. T-RCM-applaggin was prepared by treatment of 0.2 mg of RCM-applaggin with 2% (wt/wt) trypsin for 4 hr at 37°C. As with chymotryptic peptides, T-RCM-applaggin fragments were purified by reverse-phase HPLC.

Inhibition of Platelet Aggregation and Release. Platelet function studies were performed on PRP prepared from six healthy volunteers who had not taken any medications known to alter platelet activity for 2 weeks prior to blood collection. All studies were approved by an institutional review board and informed consent was obtained prior to venipuncture. Platelet counts in PRP were monitored but not adjusted (357,000 ± 56,000 counts per μl; mean ± SD; n = 6). Platelet aggregation studies were performed in a four-channel platelet aggregation profiler (PAP_4; Biodata). Inhibition of platelet aggregation by applaggin was studied by addition of protein or saline to a stirred suspension of human platelets in plasma 1 min prior to addition of agonist.

The effects of applaggin on platelet TXA_2 generation were measured by addition of the inhibitor or saline to a stirred suspension of PRP at 37°C. After 1 min, collagen (1–10 μg/ml), ADP (2–10 μM), γ-thrombin (10–20 μg/ml), or arachidonic acid (1 mM) was added to the suspension. The reaction was terminated 4 min after collagen addition with ice-cold indomethacin (10 μM), cooling to 4°C, and centrifugation at 12,000 × *g* for 2 min. The supernatant plasma was aspirated and stored at −20°C prior to measurements of the stable metabolite of TXA_2, TXB_2, by specific radioimmunoassay (22). In the absence of collagen or applaggin, the level of TXB_2 detected was <0.5 ng per 10^8 platelets.

The effects of applaggin on platelet aggregation and [^{3}H]serotonin release were measured concomitantly in PRP. Platelet count of the PRP was adjusted to 250,000 platelets per μl, and, then, 2 μl of [^{3}H]serotonin creatinine sulfate (13.5 Ci/mmol; 1 Ci = 37 GBq) was added per ml of PRP and incubated for 30 min at 37°C. [^{3}H]Serotonin-loaded platelets were kept at 37°C, continuously stirred, and stimulated by addition of ADP (2 and 10 μM), human γ-thrombin (5, 10, and 20 μg/ml), or collagen (10 μg/ml) 1 min after incubation of platelets with applaggin (0–2.5 μg/ml). [^{3}H]Serotonin release induced by each agonist was determined as percent release:

$$\% \text{ release} = 100 \times \frac{(\text{cpm/50 } \mu\text{l supernatant sample}) - (\text{cpm/50 } \mu\text{l PRP control})}{(\text{total cpm/50 } \mu\text{l PRP}) - (\text{cpm/50 } \mu\text{l PRP control})}$$

Binding of ^{125}I-Labeled Applaggin (^{125}I-Applaggin) to Platelets. Plasma-free platelet suspensions were prepared by the albumin density-gradient technique of Walsh *et al.* (23) with modifications described by Trapani-Lombardo *et al.* (24) and Ruggeri *et al.* (25). Plasma-free platelet suspensions (final concentration, 2 × 10^8 platelets per ml) were supplemented with Ca^{2+} and Mg^{2+} (final concentration, 2 mM each) and various amounts (final concentration, 0.01–0.20 μg/ml) of ^{125}I-applaggin [prepared by the Iodo-Gen method (26)] for 30 min at room temperature. Platelets and bound ^{125}I-applaggin were separated from free ^{125}I-applaggin by centrifugation of triplicate samples through a layer of 20% (wt/vol) sucrose in Tyrode's buffer containing 2% bovine serum albumin. The tip of the tube was amputated and the platelet-associated radioactivity was measured. Nonspecific binding was determined by incubating the platelets with excess unlabeled applaggin for 30 min prior to incubation with various amounts of ^{125}I-applaggin and was found at 1.0–1.5% of the total ^{125}I-applaggin bound. Binding isotherms were analyzed via the LIGAND program (27), assuming one class of noninteracting

binding sites. To study the effect of Arg_8-Gly-Asp-Val (M_r, 1539) on the binding of ^{125}I-applaggin to platelets, platelets were incubated with the peptide (final concentration, 200 μM) for 5 min at room temperature prior to incubation with ^{125}I-applaggin under the conditions described above.

RESULTS

Purification and Characterization. Applaggin was purified from the venom of the North American water moccasin snake in three steps. First, crude venom was fractionated by molecular-sieve chromatography on a column of Sephadex G-50. Contaminating protein was removed by adsorption on a cation-exchange column, and anti-platelet activity was recovered in the effluent. Applaggin was purified to >90% purity by reverse-phase HPLC performed on protein recovered from the cation-exchange flow-through (Fig. 1). Applaggin was found to elute as a sharp peak at 12% acetonitrile in the HPLC gradient and to be well-resolved from other protein contaminants. In addition, a smaller peak eluting at 13% acetonitrile was found to contain activity and to share at least the identical 41 NH_2-terminal residues with applaggin. Purification of applaggin by this method yields 3 mg of protein per g of venom.

SDS/PAGE analysis of purified applaggin (Fig. 1 *Inset*) reveals that, under nonreducing conditions, the protein has an apparent mass of 17.7 kDa. In the presence of reductant, applaggin migrates with an apparent mass of 9.8 kDa. Amino acid analysis of applaggin and RCM-applaggin reveals a high content of cysteine (21.1%) and proline (9.2%). UV absorption spectra of purified, intact applaggin revealed the absence of maximal absorbance at 280 nm and, thus, an apparent lack of tryptophan in the protein.

Sequence Analysis. The complete amino acid sequence of applaggin (Fig. 2) was obtained by NH_2-terminal sequence analysis of intact or RPE-protein and automated Edman degradation of tryptic or chymotryptic peptides derived from RCM-applaggin. NH_2-terminal sequence analysis of intact applaggin allowed determination of residues 1–30. Analysis of RPE-applaggin yielded the sequence of residues 1–61 in a single sequence run and confirmed residues 1–30. Residues 51–67 of applaggin were independently sequenced by analysis of a tryptic peptide (Gly-51 → Arg-67) derived from RCM-applaggin. A chymotryptic peptide (Cys-58 → Phe-71) derived from RCM-applaggin was used to confirm and determine amino acids from residues 58–70. A tryptic peptide derived from cleavage at the Arg-67 – Asn-68 bond in RCM-applaggin allowed determination of the COOH-terminal four residues.

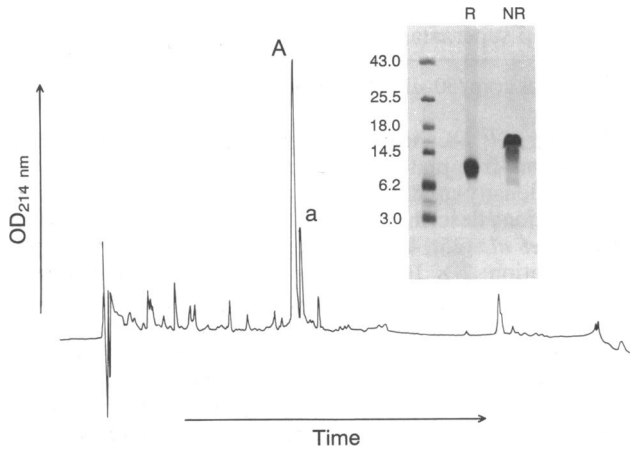

FIG. 1. Purification of applaggin by reverse-phase HPLC and SDS/PAGE analysis of purified protein. Applaggin (A) was purified by reverse-phase HPLC. The peak eluting just after applaggin (a) contains the same 41 NH_2-terminal amino acid residues as applaggin. Purified applaggin was subjected to SDS/PAGE (19% acrylamide) in the presence (R) or absence (NR) of 2-mercaptoethanol (*Inset*). The molecular masses of intact and reduced applaggin were calculated as 17.7 and 9.8 kDa, respectively.

This sequence analysis shows the applaggin protomer to be a polypeptide of 71 amino acids, which contains a high content of cysteine—namely, 12 mol of cysteine per mol of protein. Reaction of intact or denatured applaggin with 5,5′-dithiobis(2-nitrobenzoic acid) reveals the absence of accessible free thiol (J.M.M., unpublished results). Applaggin would appear to be a dimer of identical subunits linked by at least two disulfide bridges, since (*i*) SDS/PAGE analysis of applaggin shows that the protein is a disulfide-linked dimer (Fig. 1); and (*ii*) sequence analysis has revealed the presence of a single polypeptide chain with an even number of cysteinyl residues (Fig. 2).

Inhibition of Platelet Aggregation and Secretion. The dose-dependent inhibition by applaggin of collagen-, ADP-, and arachidonic acid-induced platelet aggregation was studied. Results for collagen-induced aggregation are shown in Fig. 3. Complete inhibition of collagen (1 μg/ml)- and ADP (5 μM)-induced aggregation is achieved at concentrations of applaggin at 0.6 μg/ml. Full inhibition of arachidonate (1 mM)-induced aggregation was achieved at 0.3 μg/ml concentrations of protein. In addition to inhibition of platelet aggregation, applaggin was found to inhibit generation of TXA_2 (Fig. 3) induced by collagen, ADP, γ-thrombin, and arachidonic acid. The effects of applaggin on dense granule release were studied by monitoring the inhibition of platelet serotonin release. Applaggin exhibited a dose-dependent inhibition of serotonin release induced by ADP (2 and 10 μM), γ-thrombin (5, 10, and 20 μg/ml), and collagen (10 μg/ml) (Fig. 3). Stirring of the platelet suspension was found necessary for applaggin-dependent inhibition of release (J.M.M., unpublished results). Molar IC_{50} values for applaggin inhibition of aggregation, TXA_2 generation, and dense granule release are provided in Table 1.

Binding of ^{125}I-Applaggin to Unstimulated Platelets. Scatchard analysis of the steady-state binding of radiolabeled applaggin to unstimulated platelets showed that platelets bound ≈28,500 molecules per platelet at saturation with $K_d = 1.22 \times 10^{-7}$ M. Binding of ^{125}I-applaggin to resting platelets was inhibited by Arg_8-Gly-Asp-Val at a concentration of 200 μM. The synthetic peptide Arg_8-Gly-Asp-Val has been previously shown to inhibit fibrinogen binding to platelets at concentrations similar to the dissociation constant of intact fibrinogen (28). In contrast, the synthetic peptide Arg-Gly-Asp-Val at 200 μM had no inhibitory effect on the binding of ^{125}I-applaggin (results not shown), presumably because of its lower binding affinity (28).

DISCUSSION

Snake venoms are known to contain factors that aggregate platelets directly or inhibit agonist-induced platelet aggregation (for review, see ref. 29). These factors exhibit a striking diversity in their apparent modes of action. Of the venom factors that inhibit platelet aggregation, the best described derive from *Trimeresurus gramineus* and *Echis carinatus* and are called trigramin (18) and carinatin (30) or echistatin (31), respectively. We report here on the identification, structure, and function of an anti-platelet factor, called applaggin, from the venom of *A. p. piscivorus*, the North American water moccasin snake. SDS/PAGE analysis of applaggin reveals a protein of 17.7 kDa under nonreducing conditions and of 9.8 kDa in the presence of 2-mercaptoethanol. This result suggests that unlike trigramin and echistatin, applaggin is a disulfide-linked dimer with subunits of identical size.

Amino acid sequence analysis of applaggin (Fig. 2) reveals a single polypeptide of 71 amino acids rich in cysteinyl

```
                1                                   10                                  20
APPLAGGIN:  GLU-ALA-GLY-GLU-GLU-CYS-ASP-CYS-GLY-SER-PRO-GLU-ASN-PRO-CYS-CYS-ASP-ALA-ALA-THR-
Trigramin:  Glu-Ala-Gly-Glu-Asp-Cys-Asp-Cys-Gly-Ser-Pro-Ala-Asn-Pro-Cys-Cys-Asp-Ala-Ala-Thr-
Echistatin:

                                    30                                  40
APPLAGGIN:  CYS-LYS-LEU-ARG-PRO-GLY-ALA-GLN-CYS-ALA-GLU-GLY-LEU-CYS-CYS-ASP-GLN-CYS-LYS-PHE-
Trigramin:  Cys-Lys-Leu-Ile-Pro-Gly-Ala-Gln-Cys-Gly-Glu-Gly-Leu-Cys-Cys-Asp-Gln-Cys-Ser-Phe-
Echistatin:                         Glu-Cys-Glu-Ser-Gly-Pro-Cys-Cys-Arg-Asn-Cys-Lys-Phe-
                                     1                                  10

                                                  50                                  59
APPLAGGIN:  MET-LYS-GLU-GLY-THR-VAL-CYS-(-)-ARG-ALA-ARG-GLY-ASP-ASP-VAL-ASN-ASP-TYR-CYS-ASN-
Trigramin:  Ile-Glu-Glu-Gly-Thr-Val-Cys Arg-Ile-Ala-Arg-Gly-Asp-Asp-Leu-Asp-Asp-Tyr-Cys-Asn-
Echistatin: Leu-Lys-(-)-Gly-Thr-Ile-Cys-Lys-Arg-Ala-Arg-Gly-Asp-Asp-Met-Asp-Asp-Tyr-Cys-Asn-
                                    20                                  30

                60                                  70
APPLAGGIN:  GLY-ILE-SER-ALA-GLY-CYS-PRO-ARG-ASN-PRO-PHE-HIS
Trigramin:  Gly-Arg-Ser-Ala-Gly-Cys-Pro-Arg-Asn-Pro-Phe-His
Echistatin: Gly-Leu-Thr-Cys-Asp-Cys-Pro-Arg-Asn-Pro-His-Lys-Gly-Pro-Ala-Thr
                                    40
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FIG. 2. Amino acid sequence of applaggin and comparison to trigramin (32), and echistatin (31) structures. (−), Gap inserted in the amino acid sequences to maximize sequence homology. The Arg-Gly-Asp sequence in the three venom proteins is underlined.

residues. An even number of cysteines—namely, 12 mol of cysteine per mol of protein—are found distributed throughout the polypeptide chain and are found oxidized. Together, these data suggest that applaggin is a homodimer with at least two interchain disulfide bridges. The sequence of applaggin is highly homologous to that reported for trigramin (32) and requires only a single gap in the former between residues 47 and 48 for optimal alignment (Fig. 2). The percentage identity between applaggin and trigramin is 83.3%. Residues 1–45 of echistatin align with residues 28–71 of applaggin with 54.2% identity. Placement of half-cysteinyl residues in applaggin and trigramin is conserved and, thus, a dimeric structure for applaggin requiring at least two disulfide bonds is unexpected.

A highly conserved feature in applaggin, trigramin, and echistatin is the Arg-Gly-Asp sequence, reminiscent of structure defined in fibronectin and fibrinogen, which is known to serve as one of two recognition sites for platelet binding and cross-linking via GPIIb/IIIa (4–6). As applaggin is a disul-

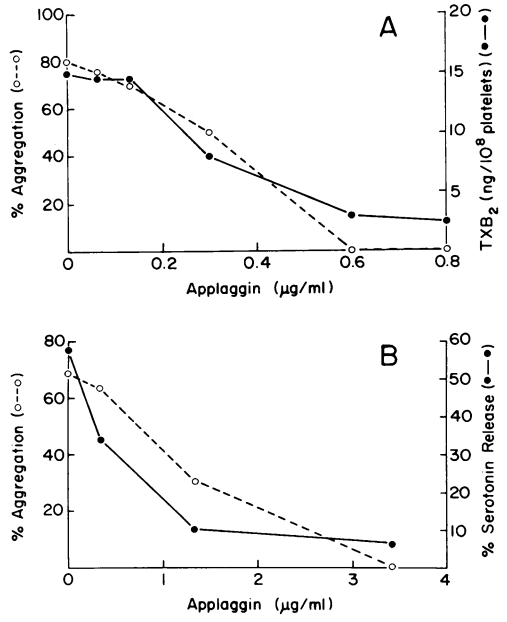

FIG. 3. Effects of applaggin on platelet aggregation, TXA_2 generation, and [^{3}H]serotonin release. Platelet aggregation studies were performed by addition of applaggin or saline to PRP 1 min prior to addition of agonist. Values for percent aggregation are reported as mean (SEM < 10%) (n = 3). (*A*) Inhibition of collagen (1 μg/ml)-induced aggregation and TXA_2 generation. TXA_2 generation was measured by radioimmunoassay for TXB_2, the stable degradation product of TXA_2. Mean values for TXA_2 generation are given (n = 3). (*B*) Inhibition by applaggin of aggregation and [^{3}H]serotonin release induced by human γ-thrombin (10 μg/ml). Percentage release was determined.

Table 1. IC_{50} values for inhibition by applaggin of platelet activation

Agonist	IC_{50}, nM		
	For aggregation	For TXA_2 generation	For serotonin release
ADP			
2 μM	17	14	<10
5 μM	17	14	—
10 μM	50	14	11
Arachidonate			
1 mM	12	13	—
γ-Thrombin			
5 μg/ml	39	—	<10
10 μg/ml	64	56	20
20 μg/ml	128	1100	>1100
Collagen			
1 μg/ml	21	17	—
10 μg/ml	89	145	145

Platelet activation studies were performed with plasma suspensions of human platelets at 37°C with constant stirring. Aggregation was monitored with an aggregometer as increase in light transmission. TXA_2 generation was measured by radioimmunoassay for TXB_2. Serotonin release was assessed with [^{3}H]serotonin-loaded platelets. That concentration of applaggin (nM) inhibiting platelet responses to a given concentration of agonist by half the maximum (IC_{50} values) was obtained from dose–response studies in which applaggin was added to PRP 1 min prior to addition of stimulus.

fide-linked homodimer, containing two Arg-Gly-Asp sequences, inhibitory effects toward platelet aggregation differ from those observed with divalent albumin conjugates of a human fibrinogen-derived dodecapeptide (Cys-γ-400–411), which promoted aggregation of ADP-stimulated gel-filtered platelets (33). Indeed, given the structural features in applaggin, one would expect the venom protein to stimulate, not inhibit, platelet aggregation. Nevertheless, applaggin is a potent inhibitor of platelet function and, thus, provides a unique example in which a divalent Arg-Gly-Asp structure can mediate such effects. Spacing of Arg-Gly-Asp sequences in the applaggin dimer may suit binding to clustered GPIIb/IIIa receptors on the same platelet surface (34).

The presence of an Arg-Gly-Asp sequence in applaggin would appear to confer platelet binding properties via interaction with GPIIb/IIIa. Affinity of applaggin for platelet is comparable to that determined for monoclonal antibodies toward GPIIb/IIIa (3, 8) and that reported for trigramin (18). The stoichiometry of ^{125}I-applaggin binding to resting platelets correlates closely with that determined for ^{125}I-fibrinogen binding to stimulated platelets reported in the literature (28), assuming that applaggin binding to platelets is bivalent per mol of protein—i.e., 1 mol of protein binds to two sites per platelet. Also, applaggin binding was inhibited in the presence of the peptide analog Arg_8-Gly-Asp-Val.

Applaggin is a potent inhibitor of platelet activation—i.e., it blocks aggregation, icosanoid metabolism, and granular release in platelets after agonist stimulation. The venom protein blocks platelet aggregation induced by all agonists tested to date with IC_{50} values ranging from 11.8 to 128 nM depending on the agonist and its concentration (Table 1). Inhibition of platelet aggregation by applaggin may well derive via interruption of fibrinogen binding to GPIIb/IIIa. It is noteworthy, however, that applaggin's inhibitory activity is substantially greater than that observed with small Arg-Gly-Asp peptides (7). Accordingly, it is likely that the conformation of Arg-Gly-Asp and its bivalent orientation found in applaggin yields high-affinity interactions with GPIIb/IIIa absent in synthetic derivatives.

In addition to interrupting platelet aggregation, applaggin inhibits both TXA_2 generation and dense granular release (Table 1). These properties, distinct from other polypeptide antagonists of the fibrinogen receptor reported to date (3, 10, 18), are observed at concentrations of applaggin comparable to those required for inhibition of platelet aggregation. The mechanism for inhibition of platelet TXA_2 generation and dense granule release by applaggin is as yet unknown. This property may relate to the divalent Arg-Gly-Asp structure in applaggin. Since platelet secretion is enhanced by close cell contact (35), applaggin-mediated inhibition of serotonin and ATP release and of TXA_2 generation may follow from interruption of platelet–platelet interactions and abrogation of the localized, synergistic, and stimulatory action of released platelet products. Determination of the mechanism for applaggin-mediated inhibition of platelet release and its possible relationship to dimeric structure in the protein may prove important in understanding the role of GPIIb/IIIa and/or other surface glycoproteins in platelet activation reactions beyond the aggregation response.

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