

Aegyptin, a Novel Mosquito Salivary Gland Protein, Specifically Binds to Collagen and Prevents Its Interaction with Platelet Glycoprotein VI, Integrin $\alpha 2 \beta 1$, and von Willebrand Factor*

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Blood-sucking arthropods have evolved a number of inhibitors of platelet aggregation and blood coagulation. In this study we have molecularly and functionally characterized aegyptin, a member of the family of 30-kDa salivary allergens from *Aedes aegypti*, whose function remained elusive thus far. Aegyptin displays a unique sequence characterized by glycine, glutamic acid, and aspartic acid repeats and was shown to specifically block collagen-induced human platelet aggregation and granule secretion. Plasmon resonance experiments demonstrate that aegyptin binds to collagen types I–V ($K_d \approx 1$ nM) but does not interact with vitronectin, fibronectin, laminin, fibrinogen, and von Willebrand factor (vWf). In addition, aegyptin attenuates platelet adhesion to soluble or fibrillar collagen. Furthermore, aegyptin inhibits vWf interaction with collagen type III under static conditions and completely blocks platelet adhesion to collagen under flow conditions at high shear rates. Notably, aegyptin prevents collagen but not convulxin binding to recombinant glycoprotein VI. These findings suggest that aegyptin recognizes specific binding sites for glycoprotein VI, integrin $\alpha 2 \beta 1$, and vWf, thereby preventing collagen interaction with its three major ligands. Aegyptin is a novel tool to study collagen-platelet interaction and a prototype for development of molecules with antithrombotic properties.

Blood-sucking arthropod saliva is a rich source of molecules that affect hemostasis (1), including vasodilators (2, 3) and inhibitors of blood coagulation (4, 5) and platelet aggregation (6–8). Among the platelet inhibitors, salivary lipocalins bind to and remove pro-aggregatory amines such as ADP (8), epinephrine, and serotonin (9, 10), whereas RGD-containing peptides block integrin $\alpha_{IIb} \beta_3$ interaction with fibrinogen (7). In addition,

enzymes such as apyrases and lipid acetylhydrolases degrade biologically active molecules such as ADP and platelet-activating factor, respectively (11, 12). Furthermore, specific antagonist of collagen-induced platelet aggregation/adhesion have been found in salivary glands of ticks and other hematophagous animals such as leeches (13–15).

Most salivary components described above have been identified through classical procedures where a given function was used to isolate an active molecule (1). Transcriptome and proteomics analyses of salivary glands also allow investigators to assign functions to arthropod saliva compounds based on sequence similarities (16–19). For example, Ixolaris from *Ixodes scapularis* was initially identified by its sequence similarity to tissue factor pathway inhibitor and shown to display a potent anticoagulant activity *in vitro* (5) and antithrombotic effects *in vivo* (20); however, as a result of this high throughput approach, a number of salivary gland transcript-encoded products have no similarity to proteins deposited in data bases. Accordingly, annotation and functional assignment for these proteins has proven difficult (1). One typical example of this complex picture is the family of 30-kDa salivary allergen found in different blood-sucking arthropods including *Aedes* sp. (17), *Culex* sp. (19), and *Anopheles* sp. (16, 18, 21, 22). Whereas these proteins are major salivary components that display significant sequence similarity, and despite several papers devoted to understanding their functional and physicochemical properties (16–19, 21, 22), their function has remained elusive thus far. In this study we show that a member of this family, herein named aegyptin, is a specific ligand of collagen.

Collagen is a matrix protein that plays a pivotal role in the process of primary hemostasis; at sites of vascular injury, it initiates recruitment of circulating platelets and triggers platelet activation cascade required to stimulate thrombus growth (23–25). The first step in platelet recruitment to collagen occurs indirectly via binding of platelet glycoprotein (GP)² Ib to collagen-bound von Willebrand factor (vWf), which plays a critical role in tethering of platelets at high shear levels (26, 27). The

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² The abbreviations used are: GP, glycoprotein; vWf, von Willebrand factor; SPR, surface plasmon resonance; BSA, bovine serum albumin; RU, resonance units; MES, 4-morpholineethanesulfonic acid.

rapid off-rate of GPIb-vWf interactions results in platelet translocation at the site of injury, allowing adhesive interactions with slower binding kinetics, such as the platelet collagen receptors glycoprotein VI and $\alpha_2\beta_1$ integrins, to mediate platelet adhesion and activation (23–28). The relative contributions of these two receptors to collagen-mediated platelet responses are under intense investigation (28–34), and different models have been proposed in an attempt to explain how platelets are activated by collagen (23, 28, 35).

Continuing our studies on antihemostatic components of blood-sucking arthropod saliva, we have assigned a function for aegyptin expressed in salivary glands of the yellow fever vector, *Aedes aegypti*. Aegyptin binds to collagens (I–V) and interferes with its interaction with major physiological ligands as follows: GPVI, integrin $\alpha 2\beta 1$, and vWf. Notably, aegyptin blocks GPVI interaction with collagen and inhibits platelet aggregation and adhesion. This study is the first to identify a collagen-binding protein in the saliva of a blood-sucking arthropod.

MATERIALS AND METHODS

Horse tendon insoluble fibrillar (quaternary, polymeric structure) Horm collagen (Chrono-Log Corp., Haverstown, PA) composed of collagen I (95%) and III (5%) was used because this microscopically visible collagen is routinely employed in platelet aggregation studies and in shear-controlled perfusion studies. Pepsin-digested, soluble nonfibrillar (tertiary structure, triple helical) collagen type III was from BD Biosciences (San Jose, CA). ADP and phorbol myristate acetate were obtained from Sigma. Ristocetin, arachidonic acid, and ChronoLumi-reagent were from Chronolog. 9,11-Dideoxy-9 α ,11 α -methanoepoxy prostaglandin F_{2 α} (U46619) was purchased from Cayman Chemical (Ann Arbor, MI); thrombin receptor-activating peptide (TRAP) was from EMD (San Diego, CA), and thrombin was from Hematologic Technologies (Essex Junction, VT). Convulxin was purified as described (36), and GPVI was prepared as published (37).

A. aegypti Collection of Saliva and Salivary Gland Dissection—Saliva from female *A. aegypti* mosquitoes was collected by oil-induced salivation (38), and salivary glands were dissected as indicated (17).

Cloning and Expression of Aegyptin—Salivary glands of 20 female mosquitoes (nonblood-fed) were dissected. Total RNA was extracted with TRIzol reagent (Invitrogen), and specific cDNA was amplified using One-step RT-PCR kit (Qiagen, Chatsworth, CA) and the gene-specific primers aegyptin forward (5'-AGGCCCCATGCCCGAAGATGAAGAACCAG-3') and aegyptin reverse (5'-TTAGTG GTGGTGGTGGTGGT-GACGTCCTTTGGATGAAACAC-3'). These two primers were designed based on aegyptin sequence (gi 94468546) to amplify the DNA fragment encoding the mature protein and a His₆ tag before the stop codon. The PCR-amplified product was cloned into VR2001-TOPO vector (modified version of the VR1020 vector; Vical Inc., San Diego), and sequence and orientation were verified by DNA sequencing. Approximately 1 mg of plasmid DNA (VR2001-aegyptin construct) was obtained using GeneElute™ HP endotoxin-free plasmid MEGA prep kit (Sigma). The plasmid was purified through a 0.22-μm filter, and recombinant protein was produced by transfecting Free-

Style™ 293-F cells (Invitrogen) with 240 μ g of purified VR2001-aegyptin plasmid, following the manufacturer's recommendations (Invitrogen). After 72 h, transfected cell culture was harvested. Supernatant containing the secreted recombinant protein was centrifuged ($100 \times g$, 15 min), frozen, and stored at -30°C until use.

Recombinant Aegyptin Purification—293-F cells supernatant containing the recombinant protein was loaded onto a Ni²⁺ column (5-ml bed volume; Amersham Biosciences) following the manufacturer's directions. Fractions were eluted with 10, 40, and 300 mM imidazole (in 50 mM Tris, 300 mM NaCl, pH 8.0), and the fraction eluted at 300 mM was pooled and concentrated in an Amicon (10-kDa molecular weight cutoff) to 1 ml and then loaded onto a size-exclusion column (Superdex 75 HR10/30; Amersham Biosciences) using the AKTA purifier system (Amersham Biosciences). Proteins were eluted at a flow rate of 0.5 ml/min in 50 mM Tris, 150 mM NaCl, pH 7.4. Purified recombinant protein was submitted to automated Edman degradation for N-terminal sequencing. To detect purity of aegyptin, 5 μ g of purified protein was loaded in a 4–12% NU-PAGE gel (MES buffer), and the gel was stained with Coomassie Blue.

Sequence Analysis and Estimate of Aegyptin Concentration—Concentration of purified aegyptin was estimated by its absorbance at 280 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and corrected according to the molar extinction coefficient $\epsilon_{280\text{ nm}} = 5600\text{ M}^{-1}\cdot\text{cm}^{-1}$; $A_{280\text{ nm/cm}}$ (1 mg/ml) = 0.220. Other calculated parameters are as follows: M_r , 27038.09; pI 3.96. Other protein parameters were obtained on line.

Salivary Gland Fractionation—One hundred pairs of salivary gland homogenates were loaded onto a size-exclusion column (Superdex 75 HR10/30; Amersham Biosciences) using the AKTA purifier system (Amersham Biosciences). Proteins were eluted at a flow rate of 0.5 ml/min in HBS-N (10 mM HEPES, pH 7.4, 150 mM NaCl). The active fraction (detected by surface plasmon resonance, see below) containing the collagen-binding protein was further purified by ion-exchange chromatography using a MonoQ column HR 5/5 (Amersham Biosciences). Proteins were eluted with a linear gradient of NaCl (0–1 M) over 60 min at a flow rate of 0.5 ml/min. Eluted proteins were again tested for collagen binding activity as described below.

Surface Plasmon Resonance (SPR) Analysis—All SPR experiments were carried out in a T100 instrument (Biacore Inc., Uppsala, Sweden) following the manufacturer's instructions. This instrument features an integrated degasser, allowing problem-free kinetic measurements at temperatures up to 45 °C, as well as a temperature-controlled flow cell and sample compartment. The Biacore T100 evaluation software was utilized for kinetic and thermodynamic evaluation. Sensor CM5, amine coupling reagents, and buffers were also purchased from Biacore Inc. (Piscataway, NJ). HBS-P (10 mM HEPES, pH 7.4, 150 mM NaCl, and 0.005% (v/v) P20 surfactant) was used as the running buffer for all SPR experiments. All SPR experiments were carried out three times.

Immobilization and Kinetic Analysis—Soluble collagen type I or type III (30 $\mu\text{g/ml}$) in acetate buffer, pH 4.5, was immobilized over a CM5 sensor via amine coupling. The immobilization target was aimed to 1500 resonance units (RU), resulting in

a final immobilization of 1737.5 RU for collagen type I and 1613.3 RU for collagen type III. Blank flow cells were used to subtract the buffer effect on sensorgrams. Kinetic experiments were carried out with a contact time of 180 s at a flow rate of 30 $\mu\text{L}/\text{min}$ at 25 °C. Aegyptin-collagen I/III complex dissociation was monitored for 1800 s, and the sensor surface was regenerated by a pulse of 20 s of 10 mM HCl at 40 $\mu\text{L}/\text{min}$. Sensorgrams were fitted using the two-state reaction (conformational change) interaction model, and a linked reactions control experiment was carried out to confirm the multiphase binding kinetics of aegyptin-collagen I interaction.

Solution Competition Assays—Experiments were performed in an attempt to detect whether aegyptin blocks collagen interaction with GPVI. Recombinant GPVI (25 $\mu\text{g}/\text{mL}$) in acetate buffer, pH 4.5, buffer was immobilized on a CM5 sensor with a final surface density of 1753.2 RU. A blank flow cell was used to subtract any effect of buffer in the refractory index change. Then different concentrations (3.175, 6.125, 12.5, 25, and 50 $\mu\text{g}/\text{mL}$) of soluble collagen I alone (control) or previously incubated (15 min at room temperature) with 500 nM of aegyptin in HBS-P buffer were injected over immobilized GPVI for 120 s at 20 $\mu\text{L}/\text{min}$. Complex dissociation was monitored for 400 s. Sensor surface was regenerated between runs with by a 30-s pulse of glycine solution, pH 1.5. To verify that immobilized GPVI was still active after all the injection-regeneration cycles, 50 $\mu\text{g}/\text{mL}$ of collagen I was injected for 120 s at a flow rate of 20 $\mu\text{L}/\text{min}$, and the resulting sensorgram was compared with the one obtained before. Additionally, a control experiment was carried out using convulxin at different concentrations (2.5, 5, and 10 nM) incubated with buffer or saturating concentrations of aegyptin (500 nM) followed by injection of the mixture over immobilized GPVI, as described above.

Identification of Collagen-binding Protein from Salivary Gland Homogenate and Saliva—An aliquot of 10 μL obtained after size-exclusion or anion-exchange chromatographies was dissolved in 100 μL of HBS-P (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% surfactant P-20) and injected over collagen type I and III immobilized on a CM5 sensor chip for 120 s at a flow rate of 20 $\mu\text{L}/\text{min}$. Complex dissociation was monitored for 400 s, and the sensor chip surface was regenerated with a 10-s pulse of 10 mM HCl at 30 $\mu\text{L}/\text{min}$. In some experiments, saliva was used as an analyte.

Thermodynamic Analysis—Thermodynamic parameters for aegyptin-collagen type I interaction were obtained from independent kinetic experiments using the Thermo Wizard assay program. Briefly, different concentrations of recombinant aegyptin (0.1–3 nM) were injected over immobilized soluble collagen type I at 15, 20, 25, 30, 35, and 40 °C. The sample compartment was kept at 25 °C. Contact time, dissociation time, and regeneration of the sensor surface were done as described above. Resulting sensorgrams were fitted to the two-state reaction (conformational change) interaction model with local R_{max} . The association (K_a) and dissociation (K_d) rate constants, as well as the affinity constant (K_D), were obtained and fitted to a linear form of the van't Hoff and Eyring equations to estimate the ΔH and ΔS as well as ΔH^{0+} and ΔS^{0+} , respectively.

Production of Aegyptin Polyclonal Antibodies and Western Blot—Female Swiss Webster mice, 8–12 weeks old, were purchased from the Division of Cancer Treatment, NCI, National Institutes of Health. Mice were maintained in the NIAID Animal Care Facility under pathogen-free conditions. Three mice were anesthetized with 100 μL of 20 mg/mL ketamine HCl (Fort Dodge Animal Health, Fort Dodge, IA) and immunized with DNA plasmids intradermally in the right ear using a 29.5-gauge needle. DNA plasmids (1 $\mu\text{g}/\mu\text{L}$) were injected in a 10- μL volume, three times at 2-week intervals. Two weeks after the last DNA immunization, sera were collected and stored at –30 °C until use. Western blot was performed using anti-aegyptin antibodies at 1:200 dilution.

Platelet Preparation and ATP Release—Blood collection, platelet aggregation, and ATP release were performed as described (8).

vWf Binding to Collagen—Polystyrene plates were coated with 100 μL of soluble collagen type III (3 $\mu\text{g}/\text{mL}$) or a 2% (w/v) solution of bovine serum albumin (BSA) diluted in phosphate-buffered saline for 2 h at 37 °C. After washing twice with phosphate-buffered saline to remove unbound protein, residual binding sites were blocked by adding 5 mg/mL denatured BSA overnight at 4 °C. After washing three times with 50 mM Tris-HCl, 150 mM NaCl, and 0.05% (v/v) Tween 20, pH 7.4 (TBS-T), increasing concentrations of recombinant aegyptin (ranging from 0.0015 to 1.5 μM) was added to the well and incubated at 37 °C for 1 h. Wells were washed again and incubated with 3 nM of vWf factor VIII-free (Hematologic Technologies, Inc.) in TBS-T supplemented with 2% (w/v) BSA. After 1 h at 37 °C, wells were washed three times with TBS-T, and a polyclonal rabbit anti-human vWf (DakoCytomation, Glostrup, Denmark) was added (1:500 in TBS-T) and incubated for 1 h at 37 °C. After three washes with TBS-T, an alkaline phosphatase conjugate anti-rabbit IgG (whole molecule; Sigma) was added (1:10,000) and incubated at 37 °C for 45 min. Before adding the stabilized *p*-nitrophenyl phosphate liquid substrate (Sigma), wells were washed six times with TBS-T. After 30 min of substrate conversion, the reaction was stopped with 3 N NaOH, and absorbance was read at 405 nm using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). Net specific binding was obtained by subtracting optical density values from wells coated only with BSA from the total binding measured as described above. All experiments were performed in triplicate.

Platelet Adhesion Assay under Static Conditions—Coverslips (22 $\times$ 22 mm, number 0) were treated with $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (4:1) for 20 min to remove contaminants (39) followed by ultrasonic washing with deionized water and ultraviolet cleaning. Coverslips were coated with fibrillar (100 $\mu\text{g}/\text{mL}$) or soluble collagen type I (100 $\mu\text{g}/\text{mL}$) for 10 min, rinsed in deionized water, and incubated overnight with denatured BSA (5 mg/mL). Coverslips were treated with 100 μL of aegyptin (0–3 μM) for 15 min, and inhibitor was removed by inverting and touching the borders of coverslips with precision wipes (Kimberly-Clark, Ontario, Canada). Platelets (200 μL , $2 \times 10^5/\text{mL}$) were mixed with aegyptin (0–3 μM) and applied to coverslips for 45 min at room temperature followed by washing in Tyrode/BSA, and mounted for imaging. Differential interference contrast images were obtained with a Leica DMI6000 microscope (Leica Microsystems, Inc., Ban-

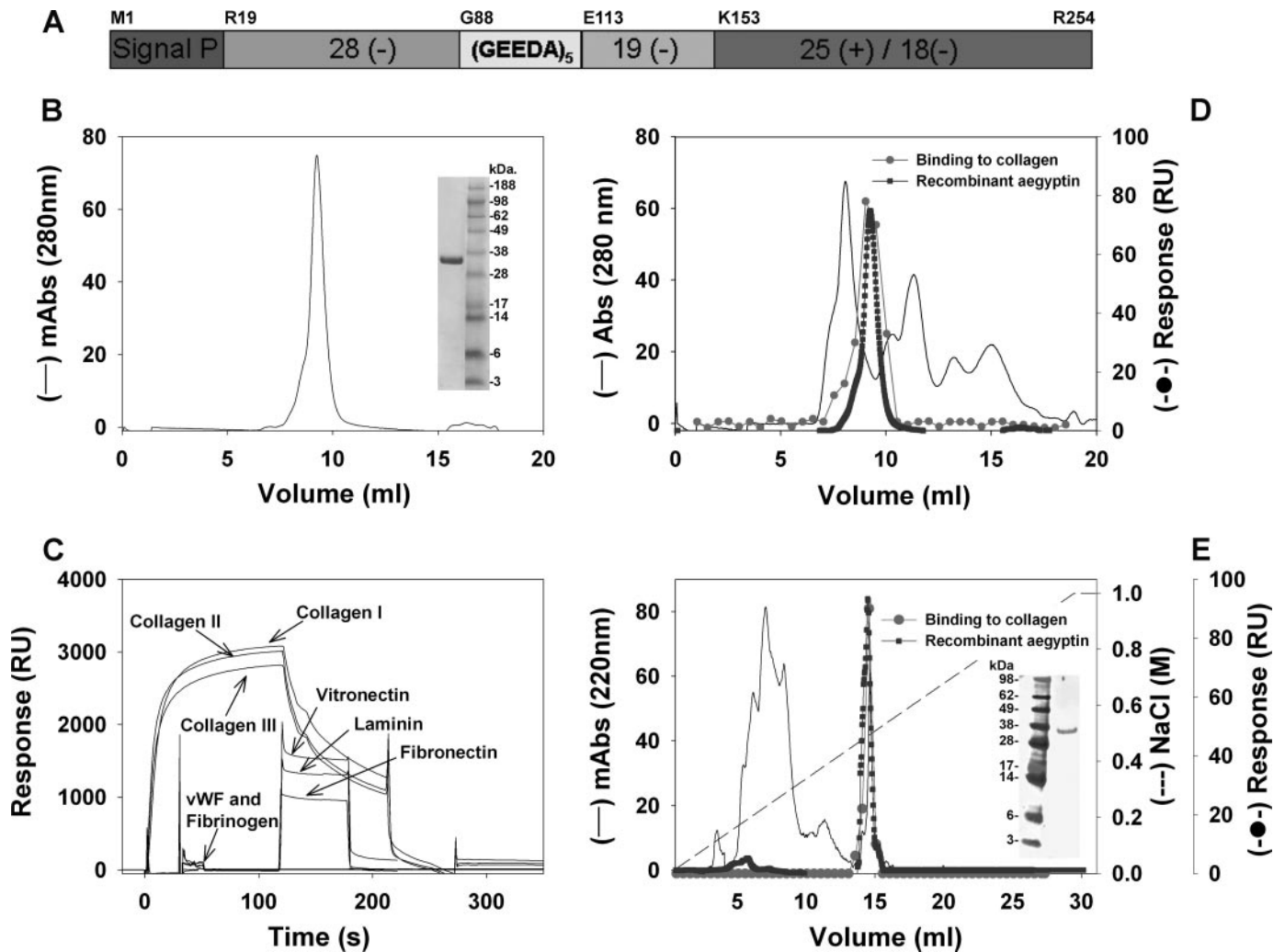


FIGURE 1. Molecular characterization of aegyptin and identification in the *A. aegypti* salivary gland. *A*, schematic representation of aegyptin with typical acidic N-terminal (GEEDA) repeats and basic C terminus depicted. *B*, purification of recombinant aegyptin after Ni^{2+} -agarose and gel filtration columns. *Inset*, NU-PAGE of purified aegyptin under denaturing conditions. *C*, plasmon resonance experiments: aegyptin interacts with collagen (I–III) but not with vitronectin, laminin, fibronectin, vWF, and fibrinogen. *D*, gel filtration of salivary gland homogenate (—) and identification of the active fraction that binds to collagen (●). The elution pattern of recombinant aegyptin is superimposed (■). *E*, anion-exchange chromatography of the active fraction obtained in *D* and identification of collagen binding activity (●). The elution pattern of recombinant aegyptin is superimposed (■). *Inset*, Western blot for detection of aegyptin in the salivary gland using polyclonal anti-aegyptin antibodies.

nockburn, IL) using $\times 100$ objective with $\text{NA} = 1.30$, and an ORCA ER digital camera (Hamamatsu Photonic Systems, Bridgewater, NJ). Image acquisition and the digital camera were controlled by ImagePro 5.1 software (Media Cybernetics, Silver Spring, MD). Extent of platelet adhesion was expressed as percent area covered by platelets.

Platelet Adhesion under Flow Conditions—Glass slides were coated with fibrillar collagen (300 μl , 100 $\mu\text{g}/\text{ml}$) for 10 min, washed in Tris-buffered saline, and incubated overnight with denatured BSA (5 mg/ml). Coated slides were treated with aegyptin (300 μl in Tyrode/BSA; 0–3 μM) for 15 min, and the excess was removed by inversion. The slides were placed in the bottom of the parallel plate flow chamber (Glycotech, Rockville, MD), and a silicone rubber gasket determined the flow path height of 254 μm as described (40). Anticoagulated blood (50 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride) was mixed with aegyptin (0–3 μM) and aspirated using an infusion/withdrawal pump with multispeed

transmission (model 940; Harvard Apparatus, Dover, MA) through the flow chamber at a flow rate of 0.65 ml/min, producing a shear rate of $1,500 \text{ s}^{-1}$ (40). Blood was perfused for 240 s followed by immediate perfusion with Tyrode/BSA (0.65 ml/min) for 120 s to remove blood, and slides were subsequently washed in Tyrode/BSA. Platelet adhesion under flow conditions was recorded using differential interference contrast imaging as described above. Extent of platelet adhesion was expressed as percent area covered by platelets.

Blood Coagulation Assays—Prothrombin time, activated partial thromboplastin time, prothrombinase, and Tenase assembly were performed as described (41).

Statistical Analysis—Results are expressed as means $\pm$ S.E.

RESULTS

Aegyptin Cloning, Expression, Purification, and Identification as a Collagen-binding Protein—Aegyptin displays sequence similarity to members of 30-kDa salivary allergens found in

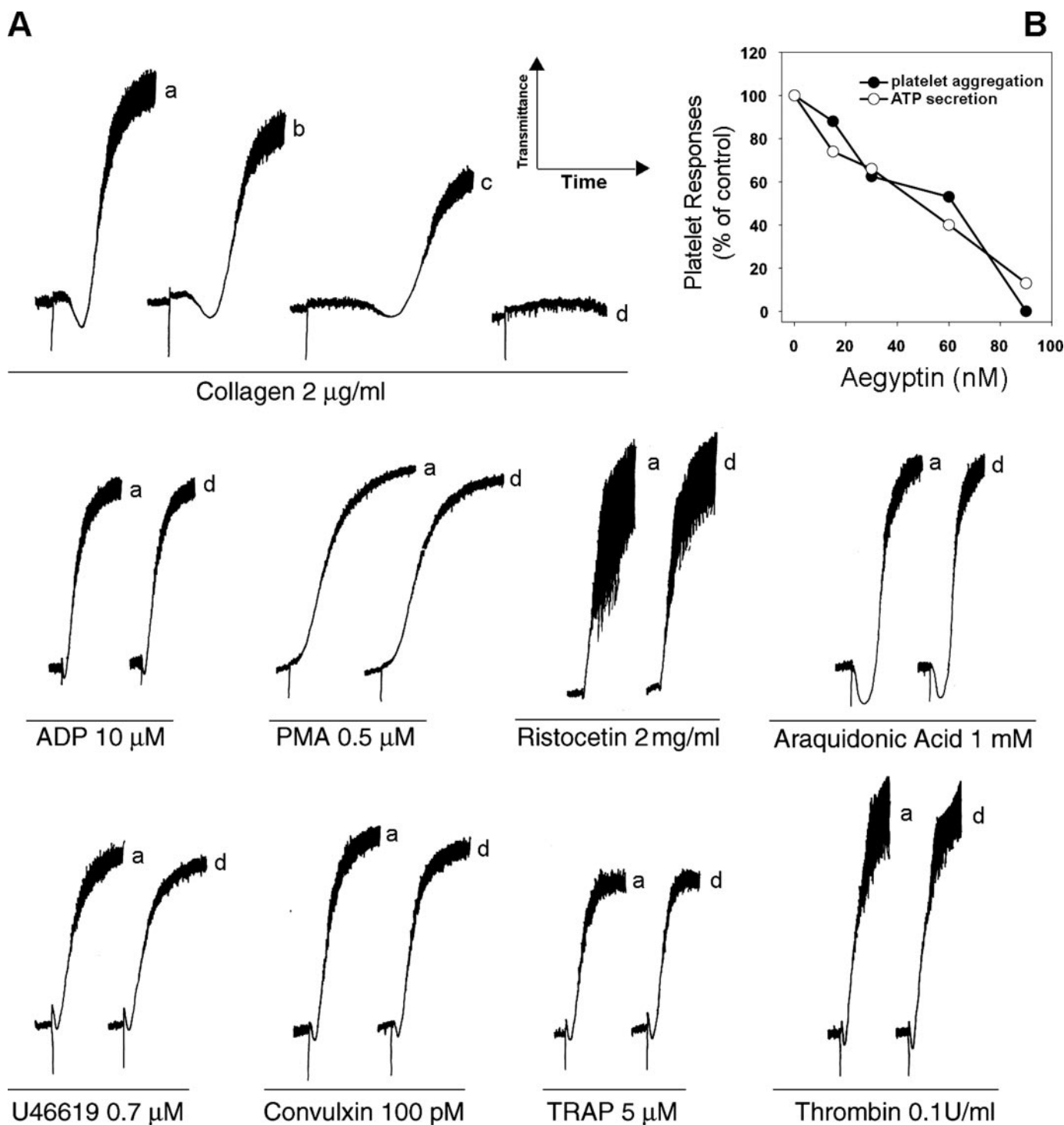


FIGURE 2. **Aegyptin specifically inhibits human platelet aggregation and granule secretion induced by collagen.** A, human platelet-rich plasma ($2 \times 10^5/\text{ml}$) was incubated with aegyptin (in nM; tracing a, 0; tracing b, 30; tracing c, 60; tracing d, 120) for 1 min followed by addition of platelet agonists as indicated. Platelet aggregation was estimated by turbidimetry under test tube stirring conditions. Washed human platelets were used when thrombin was used as an agonist. B, dose-response inhibition of collagen-induced platelet aggregation and ATP release by aegyptin. The tracings represent a typical experiment ($n = 6$).

salivary glands of blood-sucking arthropods whose function has remained elusive so far (16–19, 21, 22). Extensive sequence comparison and phylogenetic analysis have been reported for this family of proteins (17). Fig. 1A shows a diagram displaying highly acidic N terminus containing 28 negatively charged amino acids Glu or Asp and 5 Gly-Glu-Glu-Asp-Ala (GEEDA)

repeats. The repeats are followed by 19 residues of Glu or Asp and a high content of Gly. The C terminus is typically basic and display 25 positive amino acids Arg or Lys and 18 negative residues. Overall, Gly, Asp, and Glu content of aegyptin is $\approx 45\%$, whereas Arg and Lys represent 11.5% of the protein, which also displays four cysteines. In an attempt to identify the function of

TABLE 1

Kinetics of aegyptin collagen interaction

Responses were obtained by injecting recombinant aegyptin over immobilized collagen type I and III for 180 s at a flow rate of 30 $\mu\text{L}/\text{min}$.

	K_{a1} $M^{-1} s^{-1}$	K_{d1} s^{-1}	K_{a2} $M^{-1} s^{-1}$	K_{d2} s^{-1}	K_D nM	χ^2
Collagen type I	4.237×10^6	0.015400	9.818×10^{-4}	0.0193	1.22	1.26
Collagen type III	2.490×10^6	0.006055	4.889×10^{-4}	9.101	1.40	2.50

aegyptin, cDNA was cloned in a VR2001 expression vector subsequently used for transfection of 293-F cells. Medium containing the secreted recombinant protein was centrifuged, and supernatant was loaded in a Ni^{2+} column and eluted with a buffer containing increments of imidazole concentration. The protein eluted with 300 mM imidazole was further purified to homogeneity using gel filtration chromatography (Fig. 1B). Purified aegyptin was analyzed by NU-PAGE as a pure material of ≈ 30 kDa (Fig. 1B, inset), and the N-terminal obtained by Edman degradation yielded the sequence RPMPEDEEVAEG, which is in agreement with the N terminus predicted for the mature protein, according to the corresponding cDNA.

Because aegyptin has no significant matches to proteins with known function in the data bases, and considering that this protein is female-specific (17) and therefore potentially involved with blood-feeding capabilities of the mosquito, we initially carried out screening using anticoagulant assays. A series of experiments demonstrated that aegyptin does not affect coagulation tests (partial prothrombin time, prothrombin time, and thrombin time), esterolytic activity of purified enzymes (e.g. FXa, FIXa, FXIa, FXIIa, and kallikrein), and multimolecular coagulation complex assembly (extrinsic Tenase, intrinsic Tenase, and prothrombinase) (data not shown). Preliminary experiments also demonstrated that aegyptin was without effect on platelet aggregation induced by thrombin, ADP, and thromboxane A_2 mimetic (U46619); however, inhibition was observed when Horm collagen was tested as a platelet agonist suggesting that aegyptin could operate either as a platelet receptor antagonist or as collagen-binding protein. Therefore, experiments were performed to investigate whether recombinant aegyptin could directly interact with collagen using SPR experiments. Aegyptin was found to bind to soluble collagen I–III, but no interaction was observed with other matrix proteins, including laminin, vitronectin, fibronectin, vWf, and fibrinogen (Fig. 1C). It was concluded that recombinant aegyptin inhibits platelet aggregation because it specifically binds to collagen, thus preventing its interaction with platelets.

Identification of Aegyptin as a Secreted Salivary Gland Protein—To determine whether the salivary gland homogenate of *A. aegypti* contains a collagen binding activity, 100 pairs were sonicated and centrifuged, and supernatant was loaded in a gel filtration column. Fractions were tested for collagen binding activity by SPR, and the active fraction was found at a retention volume of ≈ 8.5 ml (Fig. 1D). For comparative purposes, recombinant aegyptin was applied to the same column and also eluted at ≈ 8.5 -ml retention volume. In an attempt to isolate native aegyptin from salivary glands, the active fractions obtained above were combined, concentrated, and loaded in an anion-exchange column. The active fraction was eluted at ~ 0.4 M

NaCl, which was the same salt concentration needed to elute recombinant aegyptin (Fig. 1E). In addition, Edman degradation of native aegyptin present in the active fraction identified three amino acids, Arg, Pro, and Met, which are identical to the N terminus for the mature protein as predicted by cDNA. Finally, Western blot analysis of the salivary gland homogenate using a polyclonal antibody generated by DNA vaccination identified a protein of 30 kDa whose migration pattern is identical to that of recombinant aegyptin. Therefore, we concluded that aegyptin is expressed in salivary glands of *A. aegypti* and behaves as a collagen-binding protein. In addition, both recombinant and native inhibitor display identical chromatographic and functional properties. These results validate use of recombinant aegyptin for further experimentation.

Aegyptin Is a Potent and Specific Inhibitor of Collagen-induced Platelet Aggregation—The effect of aegyptin on Horm collagen-induced human platelet aggregation was tested using test tube stirring conditions. The results show that aegyptin dose-dependently inhibits onset time for shape change and decreases the extent of platelet aggregation (Fig. 2A) and ATP secretion with an IC_{50} of ≈ 50 nM (Fig. 2B). Additionally, aegyptin (120 nM) does not affect platelet aggregation induced by ADP, phorbol myristate acetate, ristocetin, araquidonic acid, U46619, convulxin, TRAP, and thrombin (Fig. 2A).

Aegyptin Displays High Affinity Binding to Collagens—To investigate binding kinetics of aegyptin-collagen interaction, SPR experiments were performed as described under “Materials and Methods.” Typical sensorgrams obtained for recombinant aegyptin interaction with soluble collagen I and III, respectively, are shown in Fig. 3, A and B. In both cases, the best fit was attained using a two-state reaction model (Table 1), suggesting that aegyptin undergoes a conformational change after interaction with collagens. Using this model, K_d of 1.22 nM for collagen type I and K_d of 1.40 nM for collagen III were calculated (Table 1). Aegyptin also binds to soluble collagen types II, IV, V and to fibrillar collagen Type I (date not shown). Next, saliva was collected from *A. aegypti* mosquitoes, and the secretion obtained by salivation was used to verify whether it contains collagen-binding properties. Fig. 3C shows that saliva readily interacts with collagen I (sensorgram a) and III (sensorgram b). Kinetics of interaction were comparable with the pattern obtained with 0.25 nM recombinant aegyptin (Table 2). Based on these results, we confirm that aegyptin is a protein secreted in the saliva of *A. aegypti* with an estimated concentration of at least 0.3 nM in the mosquito, considering that saliva obtained by oil-induced salivation is a diluted secretion that was used for the experiments.

Thermodynamic Analysis of Aegyptin-Collagen I Interaction—Affinity and kinetic data (two-state binding model) collected at five temperatures (15, 20, 25, 30, 35, and 40 $^{\circ}\text{C}$) were fitted using Biacore evaluation software to obtain equilibrium and transi-

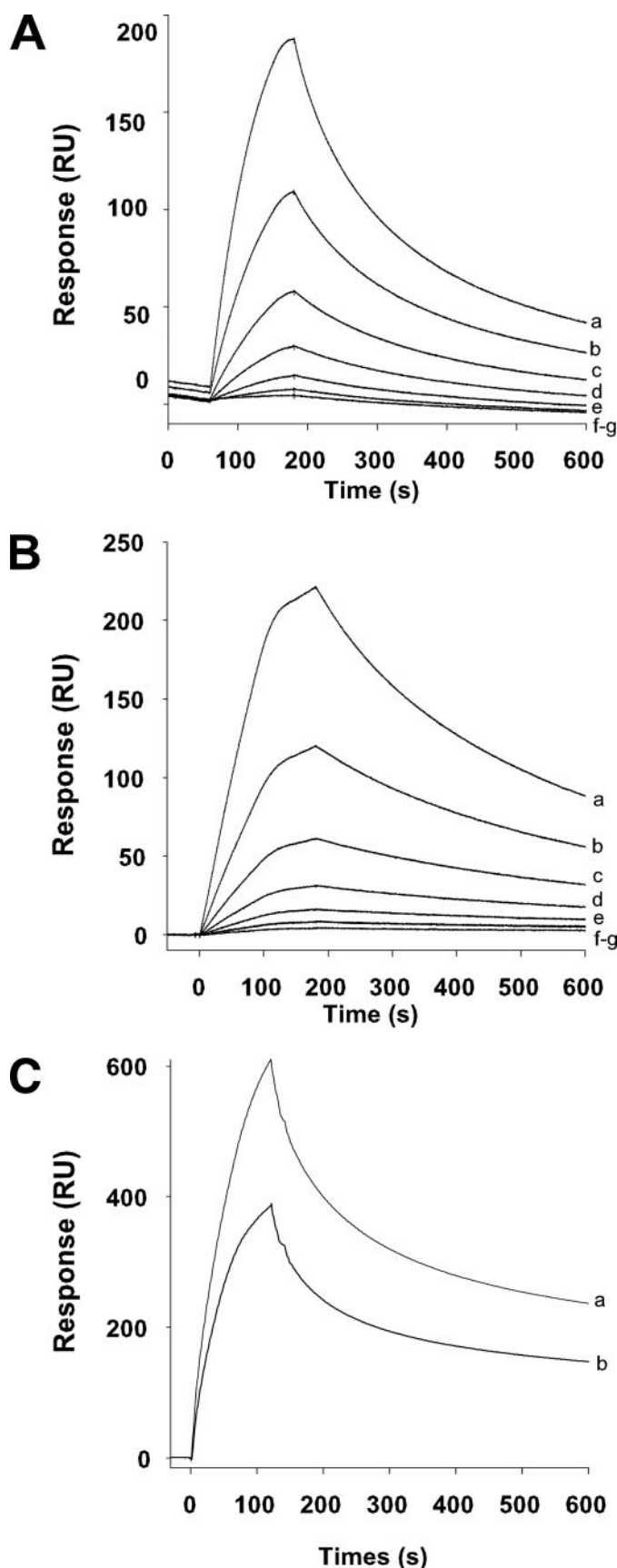


FIGURE 3. **Aegyptin binds to collagen I and III.** Typical sensorgrams of aegyptin interaction with collagen type I (A) or type III (B). Different concentrations of recombinant aegyptin (in nM: sensorgram a, 5; sensorgram b, 2.5; sensorgram c, 1.25; sensorgram d, 0.625; sensorgram e, 0.31; sensorgram f, 0.15; and sensorgram g, 0.07) were injected over immobilized collagen for 180 s. Dissociation of aegyptin-collagen complex was monitored for 1800 s, and a global two-state binding model was used to calculate kinetic parameters. C, A. aegypti saliva (0.07 µg/ml) was injected over immobilized collagen type I (sensorgram a) and III (sensorgram b) for 120 s at a flow rate of 20 µl/min. Sensorgrams are representative of triplicate experiments.

TABLE 2

Comparison between *A. aegypti* saliva and recombinant aegyptin

Responses were obtained by injecting saliva or recombinant aegyptin over immobilized collagen type I and III for 120 s at a flow rate of 20 µl/min.

	Response units	
	Collagen type I	Collagen type III
Saliva (0.07 µg/ml)	556.4	348.5
Aegyptin (0.25 nM)	590.3	397.9

TABLE 3

Thermodynamic parameters of aegyptin interaction with collagen type I

Aegyptin (0.1–3 nM) was injected over immobilized collagen type I at 15, 20, 25, 30, 35, and 40 °C. Resulting sensorgrams were fitted to the two-state reaction (conformational change) interaction model with local R_{max} .

Parameter name	Value (±S.E.)
ΔH^0 (kJ/mol)	–20 (0.85)
ΔS^0 (J/(K·mol))	95 (2.8)
$T\Delta S^0$ (kJ/mol)	28 (0.83)
ΔG^0 (kJ/mol)	–48 (0.013)

tion state thermodynamic parameters for the aegyptin-collagen interaction. The van't Hoff plot is linear over a temperature range of 15–40 °C, and the calculated free energy difference (ΔG^0) of -48 ± 0.013 kJ/mol indicates that the binding reaction occurs spontaneously. Both entropic and enthalpic components of the interaction are favorable, as indicated by a positive value for $T\Delta S^0$ (28 ± 0.83 kJ/mol) and a negative value for ΔH^0 (-20 ± 0.85 kJ/mol). This suggests that both hydrophobic and hydrogen-bonding interactions contribute significantly to the aegyptin-collagen binding reaction. Table 3 summarizes our findings.

Aegyptin Interferes with GPVI Interaction with Collagen—GPVI plays a crucial role in platelet responses to collagen and directly participates in platelet activation and supports platelet adhesion (23–25). To ascertain whether aegyptin interferes with GPVI-collagen interaction, GPVI was immobilized in a CM5 chip followed by injection of collagen I in the flow cell, previously incubated with or without inhibitor. Fig. 4A shows that increasing concentrations of collagen effectively bind to GPVI (sensorgrams a–e). Fig. 4B shows that when collagen I was incubated with buffer (sensorgram a) or increasing concentrations of aegyptin (sensorgrams c–g), collagen-GPVI interaction was abrogated only in the presence of inhibitor. As a control, sensorgram b shows that aegyptin alone does not interact with GPVI. Additional control experiments depicted in Fig. 4C demonstrate that convulxin displays high affinity binding to GPVI (sensorgrams a–c) that was not affected by high concentrations (500 nM) of recombinant aegyptin (Fig. 4D, sensorgrams a–c).

Aegyptin Interferes with Platelet Interaction with Collagen I—Integrin $\alpha 2\beta 1$ is known to mediate adhesion in a Mg^{2+} -de-

sensorgram c, 1.25; sensorgram d, 0.625; sensorgram e, 0.31; sensorgram f, 0.15; and sensorgram g, 0.07) were injected over immobilized collagen for 180 s. Dissociation of aegyptin-collagen complex was monitored for 1800 s, and a global two-state binding model was used to calculate kinetic parameters. C, A. aegypti saliva (0.07 µg/ml) was injected over immobilized collagen type I (sensorgram a) and III (sensorgram b) for 120 s at a flow rate of 20 µl/min. Sensorgrams are representative of triplicate experiments.

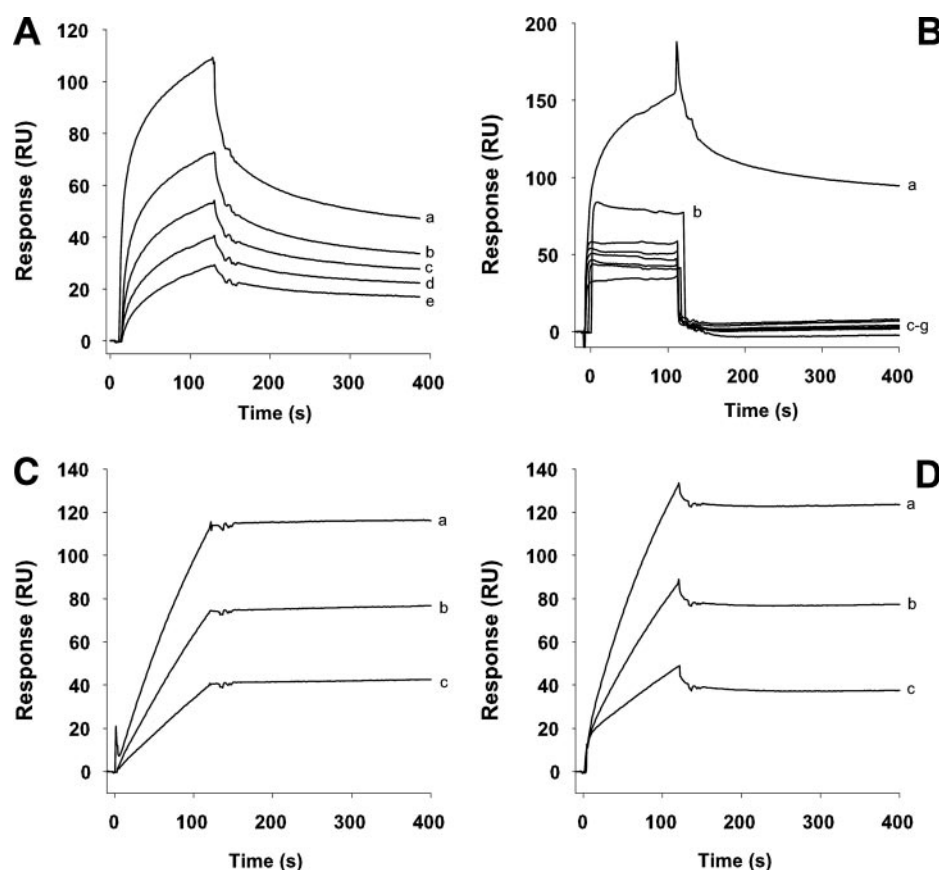


FIGURE 4. **Aegyptin inhibits collagen, but not convulxin, interaction with GPVI.** *A*, collagen type I was injected at different concentrations (in $\mu\text{g/ml}$: sensorgram *a*, 50; sensorgram *b*, 25; sensorgram *c*, 12.5; sensorgram *d*, 6.25; and sensorgram *e*, 3.175) over immobilized GPVI. *B*, collagen type I was incubated with buffer (sensorgram *a*) or 500 nM aegyptin (sensorgrams *c–g*) at the following concentrations (in $\mu\text{g/ml}$: sensorgrams *a* and *c*, 50; sensorgram *d*, 25; sensorgram *e*, 12.5; sensorgram *f*, 6.25; and sensorgram *g*, 3.175). The mixtures were injected over immobilized GPVI. Sensorgram *b* shows that aegyptin at 500 nM does not bind to immobilized GPVI. *C*, convulxin was injected at different concentrations (sensorgram *a*, 10 nM; sensorgram *b*, 5 nM and sensorgram *c*, 2.5 nM) over immobilized GPVI. *D*, convulxin (sensorgram *a*, 10 nM; sensorgram *b*, 5 nM and sensorgram *c*, 2.5 nM) was saturated with 500 nM of aegyptin, and the mixture was injected over immobilized GPVI ($n = 3$).

pendent manner. It is now recognized that the type of collagen largely determines the requirement for $\alpha 2\beta 1$. Although $\alpha 2\beta 1$ is essential for platelet adhesion and activation on monomeric type I collagen in stasis and flow, it is dispensable for these processes on native fibrillar collagen (42–44). To investigate whether aegyptin blocks integrin $\alpha 2\beta 1$ -collagen interaction, platelets were added to coverslips coated with either fibrillar or soluble collagen. Fig. 5*A* shows that aegyptin dose-dependently inhibits platelet deposition to fibrillar collagen ($\text{IC}_{50} \approx 250$ nM) (Fig. 5*B*), whereas Fig. 5*C* demonstrates that it prevents platelet adhesion to soluble collagen with an $\text{IC}_{50} \approx 200$ nM (Fig. 5*D*).

Aegyptin Interferes with vWf Interaction with Collagen III under Static and Flow Conditions—Platelet-collagen interactions are believed to have the greatest significance at the medium and high shear rates found in arteries. At the very high shear rates found in small arteries and arterioles, the rapid onset of interaction between GPIb-V-IX and vWf immobilized on collagen is crucial for initial tethering (or capture) of flowing platelets (23–26). Interaction between vWf and GPIb-V-IX, however, is rapidly reversible and insufficient for stable adhesion. At low shear rates or static condition, vWf plays a second-

ary role, but interactions can be detected using *in vitro* assays. To estimate the effects of aegyptin in vWf-collagen interaction, an ELISA assay was optimized as described under “Materials and Methods.” The results presented in Fig. 6*A* show that aegyptin dose-dependently inhibits vWf interaction with soluble collagen III with an IC_{50} of ≈ 50 nM. Next, we evaluated effects of aegyptin in platelet adhesion to collagen under flow conditions. Fig. 6*B* demonstrates that aegyptin dose-dependently inhibits platelet adhesion at shear rates of 1500 s^{-1} ; complete blockade was attained at $\approx 1 \mu\text{M}$ inhibitor. Fig. 6*C* shows a dose-response curve with an $\text{IC}_{50} \approx 300$ nM.

DISCUSSION

In an attempt to identify a function of aegyptin, a salivary 30-kDa protein of the yellow fever mosquito *A. aegypti*, the corresponding cDNA was cloned into an expression vector that was used to transfect mammalian HEK293 cells. Recombinant aegyptin was shown to specifically bind to collagen and prevent its interaction with physiological ligands GPVI, integrin $\alpha 2\beta 1$, and vWf. The fact that aegyptin affects interaction of collagen with major ligands indicates that it significantly interferes with primary hemostasis.

In fact, sub-endothelial collagen plays an important role in initiating hemostatic plug formation at sites of vascular injury, where vWf immobilized on collagen is crucial for initial capture of flowing platelets (23–26). This interaction occurs at the very high shear rates found in small arteries and arterioles but is rapidly reversible and insufficient for stable adhesion in the absence of additional platelet receptors (e.g. GPVI) (29–33) and release of pro-aggregatory mediators (e.g. ADP) (32, 45).

Because aegyptin interacts with high affinity with soluble collagen type I to V, all but type II present in the vessel wall (46), we examined whether aegyptin blocks collagen interaction with vWf. Accordingly, inhibitor dose-dependently prevents binding of vWf to soluble collagen III (47), under static conditions, with an IC_{50} of ≈ 50 nM. Most important, aegyptin completely inhibits interaction of platelets with fibrillar collagen I under high shear rate (1500 s^{-1}) (26). Accordingly, platelet deposition in collagen coverslips was severely impaired by nanomolar concentrations of inhibitor, as depicted in Fig. 6. These findings indicate that aegyptin recognizes specific or nearby sequences (47) and functionally inhibits GPIb-vWf binding to collagen under flow conditions, thus preventing subsequent events

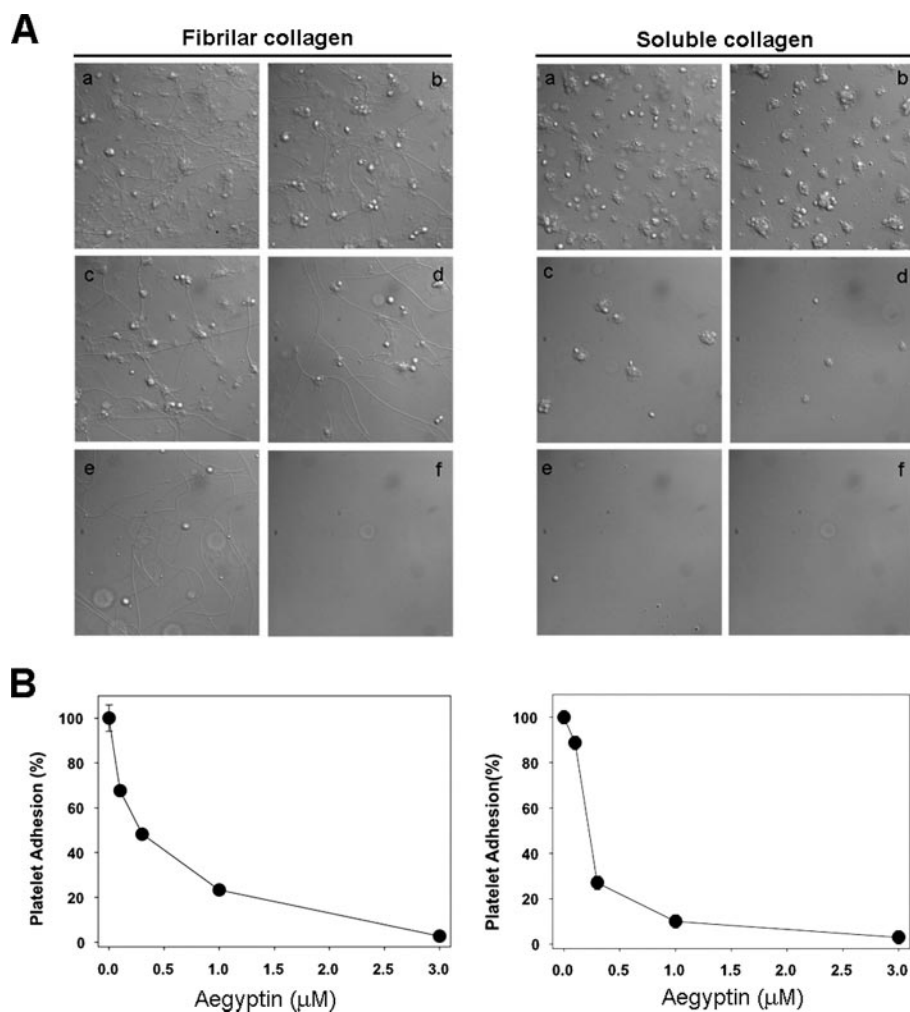


FIGURE 5. Aegyptin prevents platelet adhesion to fibrillar and soluble collagen under static conditions. Washed human platelets ($2 \times 10^5/\text{ml}$) were incubated with fibrillar (A) or soluble collagen (C) for 45 min in the presence of aegyptin at various concentrations: panel a, 0 μM ; panel b, 0.1 μM ; panel c, 0.3 μM ; panel d, 1 μM ; and panel e, 3 μM . In panel f, coverslips were coated with denatured BSA in absence of collagen. B and D are dose-response curves for aegyptin-mediated inhibition of platelet adhesion to fibrillar or soluble collagen, respectively. Typical experiments are shown ($n = 3$).

involved in normal hemostasis and/or pathologic thrombus formation *in vivo* (29–33).

After initial vWf-dependent tethering of platelets, GPVI-collagen interactions initiate cellular activation followed by shifting of integrins to high affinity state and release of second-wave agonists, most importantly ADP and thromboxane A_2 (32, 45). Cellular activation and up-regulation of integrin affinity is also proposed to be an important prerequisite for adhesion (32, 35, 45). Therefore, we investigated whether complex formation between aegyptin and collagen could prevent GPVI-mediated platelet responses. As expected, aegyptin is a potent inhibitor of collagen-induced platelet-rich plasma aggregation under test tube stirring conditions ($\text{IC}_{50} \approx 50 \text{ nM}$). This effect was accompanied by attenuation of shape change and granule secretion, suggesting that platelet-collagen interaction was fully prevented by inhibitor at appropriate concentrations. Furthermore, SPR experiments show that interaction of collagen with immobilized recombinant GPVI was inhibited by aegyptin, whereas convulxin-GPVI binding was not affected. These results indicate that aegyptin binds to specific (or nearby)

sequences that mediate collagen-GPVI interaction (48). This is a notable finding, because GPVI has been regarded as a critical receptor involved in platelet activation through a mechanism involving tyrosine phosphorylation of FcR γ chain, *syk*, *fyn*, and *lyn*, and the participation of adapter proteins such as LAT and SLP-6. This cascade of phosphorylation reactions lead to phospholipase $C\gamma 2$ activation, which is a major step necessary for collagen-induced platelet activation (49, 50).

It is important to recognize that firm adhesion of platelets to collagen through activated $\alpha 2\beta 1$ results in sustained GPVI signaling accompanied by enhanced release and procoagulant activity (51, 52). Therefore, it was of interest to ascertain whether aegyptin specifically prevents platelet adhesion to collagen mediated by integrin $\alpha 2\beta 1$ and/or GPVI. Our results unambiguously show that platelet adhesion was prevented by inhibitor under static conditions regardless of whether soluble or fibrillar collagens were tested. Because adhesion to fibrillar collagen is mediated mostly by GPVI, whereas adhesion to soluble collagen is exclusively dependent on integrin $\alpha 2\beta 1$ (42–44), it is clear that inhibitor recognizes binding sites in the collagen molecule that mediate its interaction with both receptors (48, 53). Furthermore, it is evident that aegyptin does not discriminate between fibrillar (insoluble) and nonfibrillar (soluble) collagen and binds with similar affinity to different types of collagens (I–V). It may be that the unique sequence of aegyptin characterized by high content of GDE provides an efficient template for high affinity binding to collagen through a thermodynamically favorable reaction (Table 3). Therefore, aegyptin appears to recognize the fundamental structure of the collagen molecule, which is characterized by the presence of one or more triple helical domains formed by three polypeptide α chains. Within these domains, the three α chains wind around one another in a characteristic left-handed triple helix (25). In other words, aegyptin recognizes the three-dimensional structure of collagen crucial for ligand recognition. Actually, all collagens tested to date exhibit the ability to induce platelet aggregation *in vitro* when in the appropriate polymeric form (46, 54, 55). This may reflect the presence within the triple helix of GPO triplets that interact with platelet GPVI (48, 56) and presumably so with aegyptin. Alternatively, aegyptin induces a conformational change in col-

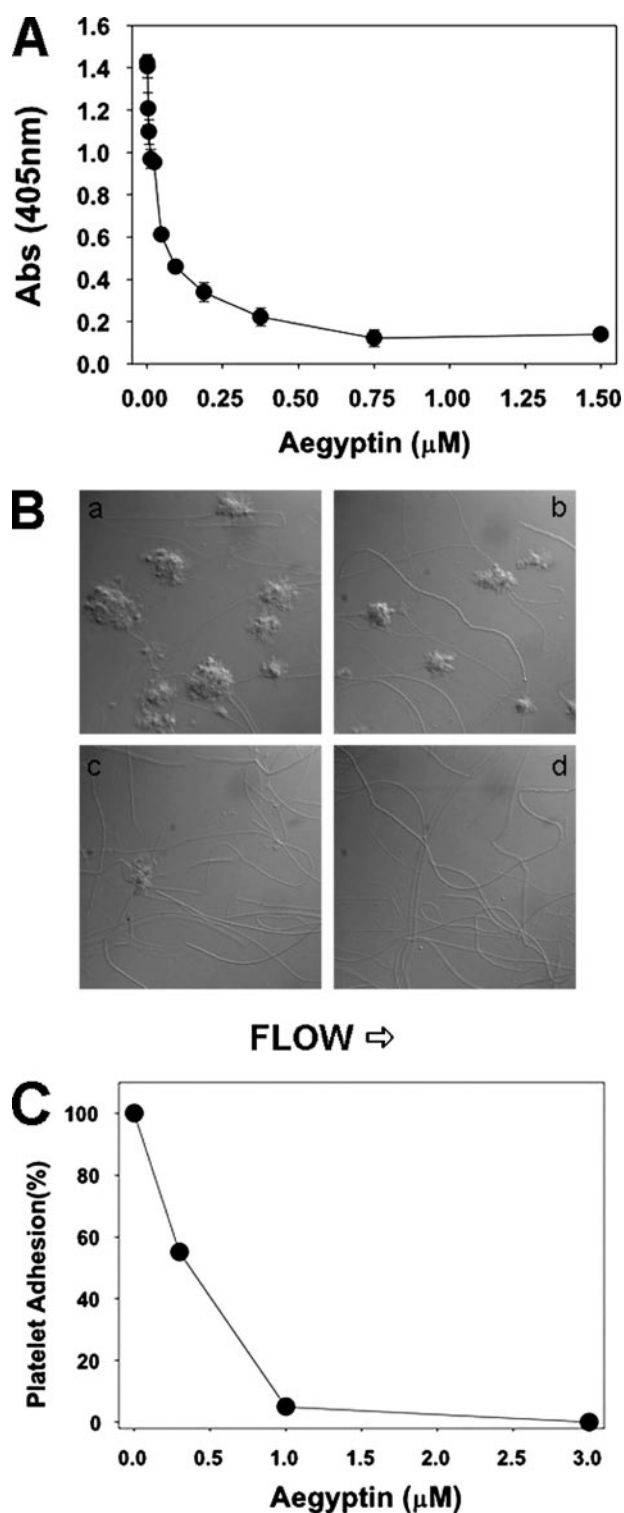


FIGURE 6. Aegyptin inhibits interaction of vWf with collagen under static and flow conditions. *A*, inhibition of vWf binding to soluble collagen III was estimated by enzyme-linked immunosorbent assay in the presence of indicated concentrations of aegyptin. *B*, anticoagulated whole blood was perfused over immobilized fibrillar collagen for 240 s at a shear rate of 1500 s^{-1} in the presence of 0 (panel *a*), 0.3 (panel *b*), 1.0 (panel *c*), and 3.0 μM (panel *d*) of aegyptin. *C*, dose-response curve for aegyptin-mediated inhibition of platelet adhesion to collagen under flow conditions. Typical experiments are shown ($n = 3$).

lagen molecule which affects its interaction with physiological ligands.

In addition to aegyptin, two leech-derived proteins, namely

LAPP (from *Haementeria officinalis*), and saratin (from *Haementeria medicinalis*) (13–15), and a plasma protein C1qTNF-related protein-1 (CTRP-1) (57) have been molecularly cloned and shown to specifically interact with collagen. Although LAPP prevents platelet aggregation and vWf binding to collagen, it marginally inhibits platelet adhesion to collagen under low shear rates (58). On the other hand, saratin effectively inhibits vWf binding but only partially affects platelet aggregation at very high concentrations (100 $\mu\text{g/ml}$) (15). Furthermore, CTRP-1 prevents platelet aggregation primarily because it blocks vWf binding to collagen (57). Therefore, each molecule distinctly recognizes binding sites in the collagen molecule, ultimately resulting in a certain degree of inhibition of platelet function as demonstrated for saratin (59), rLAPP (60), and CTRP-1 (57) when tested *in vivo*.

Our results indicate that aegyptin displays antihemostatic properties through inhibition of collagen interaction with its three major ligands and therefore mechanistically and functionally distinguishes it from other collagen-binding proteins described previously (13–15, 57). These findings are particularly relevant because the adhesive potential of platelets results from the sum of distinct pathways supported by coordinated receptor-ligand interactions specially adapted to respond to different environmental conditions (26). Also, fibrillar collagen is widely recognized as a particularly atherogenic molecule together with tissue factor (61–63). In addition, pepsin-digested collagen, which is readily recognized by aegyptin, displays pro-adhesive properties *in vitro* and possibly *in vivo* where matrix metalloprotease activity of infiltrating macrophages and activated smooth muscle cells has been reported (64). Accordingly, $\alpha 2\beta 1$ may substantially contribute to thrombus formation in synergy with other receptors GPVI/FcR γ and GPIb/IX/V, whose interaction is also prevented by aegyptin. Thus, aegyptin may be useful as a tool to study structural features of collagen in designing specific inhibitors targeting collagen interaction with GPVI, integrin $\alpha 2\beta 1$, and/or vWf.

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