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Aegyptin displays high-affinity for Willebrand Factor Binding Site (RGQOGVMGF) in Collagen and Inhibits Carotid Thrombus Formation *in vivo*

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Abstract

Aegyptin is a 30-kDa mosquito salivary gland protein that binds to collagen and inhibits platelet aggregation. We have studied biophysical properties of aegyptin and its mechanism of action. Light scattering plot shows that aegyptin displays a monomeric elongated form which explains the apparent molecular mass of 110 kDa estimated by gel-filtration chromatography. Surface plasmon resonance identified the sequence RGQOGVMGF (O is hydroxyproline) that mediates collagen interaction with von Willebrand Factor (vWF) as high-affinity ($K_D \approx 10$ nM) binding site for aegyptin. Additionally, aegyptin interacts with linear RGQPGVMGF peptide and heat-denatured collagen, implying that the triple-helix and hydroxyproline are not a prerequisite for binding. In contrast, aegyptin does not interact with scrambled RGQPGVMGF peptide. Aegyptin also recognizes with low affinity (μ M range) peptides (GPO)₁₀ and GFOGER which respectively represent glycoprotein VI and integrin $\alpha 2\beta 1$ binding sites in collagen, and prevents platelet adhesion and aggregation. Truncated forms of aegyptin were engineered, and the C-terminus fragment was shown to interact with collagen and to attenuate platelet aggregation. In addition, aegyptin prevents laser-induced carotid thrombus formation in the presence of Rose Bengal *in vivo*, without observable bleeding in rats. In conclusion, aegyptin interacts with distinct binding sites in collagen, and is useful tool to inhibit platelet-collagen interaction *in vitro* and *in vivo*.

Keywords

aegyptin; blood-sucking; hematophagy; mosquitoes; yellow fever; GPVI

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INTRODUCTION

Collagen is a triple helical protein that constitutes the major structural component of the extracellular matrix [1,2]. Damage to the blood vessel endothelium results in the exposure of fibrillar collagen I and III, both abundant in the subendothelial space. Interaction of circulating platelets with collagen is a multistage process involving several receptors and the relative contribution of each of them has been intensely investigated [3–5]. Accordingly, the initial tethering of platelet to the ECM is mediated by the interaction of platelet receptor glycoprotein (GP) Ib and von Willebrand Factor (vWF)-bound collagen, particularly at high shear rates [3–5]. This interaction allows the binding of the collagen receptor GPVI [6] to its ligand and to initiate cellular activation, a process that is reinforced by locally produced thrombin and soluble mediators released from platelets [3–5]. These events lead to the shift of integrins on the platelet surface from a low to a high affinity state, thereby enabling them to bind their ligands and to mediate firm adhesion, spreading, coagulant activity, and aggregation [7–10]. This process is crucial for normal hemostasis, but may also lead to pathological thrombus formation causing diseases such as myocardial infarction or stroke [11,12].

Exogenous secretion from snake venom and blood sucking invertebrates such as mosquitoes, ticks, and leeches are rich sources of modulators of hemostasis, and the immune system [13,14]. Recently we discovered that *Aedes aegypti* salivary gland expresses aegyptin, a potent collagen-binding protein that prevents its interaction with three major ligands, namely, GPVI, vWF and integrin $\alpha 2\beta 1$ [15]. Aegyptin displays sequence and functional similarities to AAPP, a collagen-binding protein from the salivary gland of *Anopheles stephensi* [16]. Our aim in this study has been to understand the molecular mechanism by which aegyptin interacts with collagen, and to investigate its potential antithrombotic properties. It was found that aegyptin recognizes with high affinity the sequence involved in collagen interaction with vWF, and also interacts with GPVI and integrin $\alpha 2\beta 1$ binding sites. Aegyptin effectively inhibits carotid thrombus formation *in vivo*.

Results

Aegyptin displays an elongated structure

Aegyptin is a collagen-binding protein from the salivary gland of the mosquito *Aedes aegypti*, and obtained in recombinant active form as described before [15]. The molecular mass of aegyptin (mature peptide) predicted by its primary structure is 27 kDa [17] and PAGE under denaturing conditions shows that it migrates as 30 kDa protein (Figure 1A, inset). However, it elutes at higher apparent molecular mass of 112 kDa when loaded on gel filtration column (Figure 1A), suggesting aegyptin is oligomeric or may significantly deviate from a spherical shape. Since elution time on a size exclusion column cannot distinguish between these possibilities, size exclusion chromatography with on-line multi-angle light scattering (SEC-MALS-QELS-HPLC) was used to analyze the hydrodynamic radius (R_h) of recombinant aegyptin. Multi-angle light scattering indicated the protein to elute as a monomer of 33 ± 1.67 kDa (Figure 1B) with a hydrodynamic radius of 4.8 ± 0.29 nm, indicating that aegyptin in solution is a monomeric non-globular elongated protein with a molecular mass of 33.4 kDa, providing the explanation for the anomalous retention time observed on the analytical sizing column. The elongated structure of aegyptin may favor its interaction with collagen. Next, we attempted to estimate the presence of regular secondary structure in aegyptin which can be recognized from the wavelengths of peaks in their circular dichroism spectra. Alpha helices show negative peaks at 208 and 222 nm and a positive peak at 190 nm, while beta sheets show a negative band near 220 nm and a positive band at 190 nm. Accordingly, Figure 1C shows the spectra of recombinant aegyptin which is characterized by a rich content in alpha/beta structures.

High-affinity binding of aegyptin to collagen estimated by SPR

In order to study the kinetics of aegyptin interaction with immobilized collagen by SPR, experiments were performed to optimize assay conditions, identify the appropriate equation to fit the experimental results, and to minimize mass transfer effects. Figure 2A shows surface plasmon resonance (SPR) binding kinetics collected on aegyptin interaction with collagen immobilized at relatively low density (620.8 RU) in CM5 sensor chip. Sensorgrams (black lines) display biphasic kinetics that fit best to a two-state reaction (conformational change, red line) mechanism with two on- and off-rate constants and similar K_D 5.9 ± 0.3 nM. This value displays a similar affinity calculated for aegyptin interaction with collagen immobilized at high density (1760.2 RU), with K_D 6.1 ± 0.4 nM; in both cases *chi* squares values were kept at low levels. Sensorgrams were also fitted using a 1:1 model (not shown, supplemental data, Figure S2A), and while K_D were comparable to value obtained with two-state reaction model, *chi*-squares were significantly higher. Table 1 summarizes the results. Because collagen fibers are much larger than aegyptin, it is expected that it could bind multiple aegyptin molecules. To verify this hypothesis, SPR experiments were performed where collagen was immobilized in the sensor and used to bind aegyptin. In the reverse system, aegyptin was immobilized on the sensor and collagen used as the ligand (analyte). Figure 2B shows that aegyptin binding to immobilized collagen is followed by slow dissociation phase, as described previously [15]. However, when aegyptin is immobilized, interaction with collagen is tight as often observed for bifunctional or multivalent proteins [18, 19](see Discussion).

High-affinity binding of aegyptin to collagen estimated by solid-phase binding assay and fluorescence microscopy

To estimate aegyptin binding to collagen by an additional technique, solid-phase binding assays were performed as described in Methods. Figure 2C shows that binding of aegyptin to immobilized collagen occurs in a dose-dependent and saturable manner with an apparent K_D 41.0 ± 6.9 nM. This value is in reasonable agreement with $K_D \approx 6$ nM obtained previously by SPR (Table 1), and calculated using a different set of experiments and equations.

In order to verify the pattern of aegyptin binding to collagen fiber, inhibitor was labeled with FITC and incubated with immobilized collagen as described in Methods. Figure 2D shows collagen fibers detected by bright field microscopy observed under differential interference contrast (DIC) (left, upper and lower panels) and demonstrates that aegyptin-FITC interacts with most collagen fibrils immobilized in the coverslips (right, upper panel). When PBS was used (negative control), no auto-fluorescence was detectable for collagen (lower, right panel).

Aegyptin binds with high affinity to vWF binding site in collagen, independently of hydroxyproline

In an attempt to identify the binding sites involved in collagen interaction with aegyptin, a series of peptides based on collagen sequences which reportedly mediate collagen interaction with physiological ligands were synthesized. The peptides (GPO)₁₀ [20], GFOGER [21] and RGQOGVMGF [22] were then cross-linked and used for SPR experiments and functional assays *in vitro*, as described in Methods. Figure 3A shows that aegyptin interacts with cross-linked RGQOGVMGF peptide with a calculated K_D 23.98 ± 1.67 nM. Figure 3B shows that aegyptin also binds to linear RGQOGVMGF with high affinity (K_D 41.81 ± 5.05 nM) implying that the triple helix structure is not needed for binding. Next, hydroxyproline-less RGQPGVMGF peptides were tested in SPR assays. Figures 3C and 3D show that high-affinity aegyptin-peptide interaction occur independently of hydroxyprolines residues, in cross-linked or linear peptides, respectively. Control

experiments performed in parallel using scrambled RGQPGVMGF peptide, soluble collagen III and RGQPGVMGF peptide immobilized in different flow cells of the same CM5 sensor chip demonstrated that scrambling the sequence RGQPGVMGF is accompanied by complete loss of binding to aegyptin (Figure 3E). Control experiments were also performed to verify whether the peptide was functional. Figure 3F shows that aegyptin prevents vWF interaction with RGQOGVMGF with IC_{50} 310.7 ± 25.6 nM.

Individual collagen molecules maintain their integrity by non covalent bonds, and denaturation leads to unraveling of the coiled-coil and dissociation of the three chains. Heating the collagens above a critical temperature causes denaturation, reflected in a rapid loss of the triple helical structure [1,2]. Sensorgrams shown in Figure 3G shows that aegyptin binds to heat-denatured collagen with affinity comparable to the native molecule (Figure 2A) indicating that the primary sequence is indeed sufficient for the interaction.

Aegyptin binds with low affinity to GPVI and integrin $\alpha 2\beta 1$ binding sites in collagen

Next, sequences involved in collagen interaction with GPVI and integrin $\alpha 2\beta 1$ were tested as potential binding sites for aegyptin. Figures 4A and 4B respectively show typical sensorgrams of aegyptin binding to $(GPO)_{10}$ and GFOGER; the data were fitted with a two-state binding model and yields a K_D of 9.6 ± 0.38 μ M and 2.4 ± 0.19 μ M, respectively. While aegyptin prevents collagen-induced platelet aggregation under test-tube stirring conditions with an $IC_{50} \approx 100$ nM [15] it was ineffective to inhibit $(GPO)_{10}$ -induced platelet aggregation (Figure 4C), consistent with a low-affinity interaction. Figure 4D shows that aegyptin prevents platelet adhesion to immobilized collagen in a dose-dependent manner, but was ineffective when GFOGER was immobilized, likely due to low affinity. Of note, the interaction between different peptides and collagen with aegyptin displayed biphasic binding kinetics with relatively similar $ka1$ and $ka2$ rates. On the other hand, the off rate, $kd1$ for $(GPO)_{10}$ and GFOGER interaction with the inhibitor were ≈ 100 fold faster relative to collagen, and RGQOGVMGF peptide (Table 2). These results suggest that the lower affinity of aegyptin for $(GPO)_{10}$ and GFOGER derives primarily from an accelerated $kd1$. Table 2 summarizes the kinetic findings and displays the χ -square for each interaction. The supplemental data show actual sensorgrams and corresponding fitting using 2-state reaction model for all results presented herein.

Identification of C-terminal as a functional domain of aegyptin

It was of interest to identify the aegyptin domains that account for the collagen-binding properties. A number of truncated forms or fragments corresponding to the N-terminus (1–39 aa), C-terminus 1 (113 aa), C-terminus-2 (137 aa), mid-domain (132 aa) and GEEDA repeats (50 aa) of aegyptin were expressed and purified. A diagram for each fragment is shown in Figure 5A. Among all truncated forms tested only C-terminus-2 was shown to interact with collagen (Figure 5B) with K_D 92.82 ± 4.64 nM (Figure 5C). Figure 5D shows that C-terminus-2 delays the shape change and prevents collagen-induced platelet aggregation with $IC_{50} \approx 3.0$ μ M, but not platelet aggregation triggered by 100 pM convulxin (not shown), a toxin that also activates platelets through GPVI without sharing structural features with collagen [6, 23].

Aegyptin displays anti-thrombotic activity *in vivo*

We investigated whether aegyptin displays *in vivo* antithrombotic properties using a laser induced model of carotid injury in rats [24,25]. With photochemical injury, dye (*e.g.* Rose Bengal) is infused into the circulation. Photo-excitation leads to oxidative injury of the vessel wall and subsequent thrombus formation [24]. Figure 6A shows that the blood flow of control (PBS injected) animals stopped in 19.37 ± 2.38 mins. On the other hand, the time for thrombus formation in animals treated with 50 μ g/kg aegyptin was 54.57 ± 9.44 mins, and

reproducibly delayed more than 80 minutes with 100 $\mu\text{g/kg}$. Figure 6B shows that bleeding in control animals was 25.73 ± 1.7 ml/hour after 15 min of injection of PBS; in the presence of aegyptin it increased non-significantly to 31.07 ± 4.9 $\mu\text{l/hour}$ (50 $\mu\text{g/kg}$) and 45.73 ± 7.2 $\mu\text{l/hour}$ (100 $\mu\text{g/kg}$). In the presence of heparin (1 mg/kg), bleeding augmented significantly to 62 $\mu\text{l/hour}$ with $p < 0.05$.

Discussion

This paper investigates the molecular mechanism by which aegyptin prevents platelet activation induced by collagen, a highly thrombogenic protein of the vessel wall [26–28]. Results using surface plasmon resonance (SPR), solid-phase binding assays, and fluorescence microscopy confirm that aegyptin is a collagen-binding protein [15]; results also provide evidence to conclude that aegyptin interacts primarily with the collagen sequence that mediates its interaction with vWF [22]. Accordingly, SPR and ELISA experiments respectively show that aegyptin preferentially recognizes the RGQOGVMGF sequence and blocks vWF binding to the corresponding peptide (Figure 3A and 3F). SPR experiments also suggest that aegyptin:collagen complex formation displays a more complex binding mechanism and involve a two-step reaction where an encounter complex, (aegyptin:collagen)*, is observed before reaching the final complex state. The significance of the two-step binding reaction of aegyptin:collagen interaction and the possible contribution of elongated structure of aegyptin are open questions that future studies will explore.

In agreement with SPR experiments, aegyptin prevents vWF binding to collagen in static conditions and attenuates vWF-dependent platelet adhesion to collagen under high shear rates [15]. Interestingly, the vWF binding domain in collagen has been identified as the binding site for SPARC/BM-40/osteonectin [29], discoidin domain receptor 2 (DDR2) [30], calin [31], LAPP [32], saratin [33,34], CTRP-1 [35] and atrolysin A [36] underscoring an important role for this domain in matrix interactions with structurally unrelated molecules. Our results also show that aegyptin binds with high-affinity to non-crosslinked (linear) RGQOGVMGF or RGQPGVMGF sequences and interacts with heat-denatured collagen, a molecule that is typically devoid of triple-helical structures [1,2]. In contrast, binding was not detectable when scrambled RGQPGVMGF peptide was immobilized in the sensor chip. Therefore, aegyptin specifically recognizes vWF binding site found in collagen and no minimal number of GPP/GPO stretches is necessary for complex formation. In other words, the native collagen triple-helical structure and hydroxyproline residues are not a prerequisite for aegyptin binding. Similar conclusions have been reported for keratinocyte growth factor, oncostatin M, interleukin 2 and PDGF binding to collagen which is not prevented by reduction and alkylation, or heat denaturation [37]. Of note, collagen is thermally unstable at body temperature and has been reported to display a random coil rather than triple helix structure only [38]. Further, denatured collagen modulates the function of fibroblasts and promotes wound healing suggesting that if biologically active *in vivo* [39], it would be a potential target for aegyptin.

While aegyptin binds to RGQOGVMGF, it also recognizes (GPO)₁₀ and GFOGER with lower affinity (Figure 4), and it effectively prevents GPVI interaction with collagen, blocks platelet aggregation, and attenuates integrin $\alpha 2\beta 1$ -dependent platelet adhesion [15]. It is conceivable that aegyptin interacts with GPVI and integrin $\alpha 2\beta 1$ binding motifs in native collagen with higher affinity than observed with the corresponding synthetic peptides (GPO)₁₀ and GFOGER, respectively (Figures 4A and 4B). It is also plausible that aegyptin binding to vWF-binding site in collagen sterically interferes with collagen binding to integrin $\alpha 2\beta 1$ since these sites are in close spatial proximity [40]. Alternatively, multiple low affinity interactions contribute to the high affinity observed between aegyptin and collagen,

as described for bifunctional proteins such as the thrombin inhibitors anophelin [18] and rhodniin [19]. These inhibitors recognize thrombin catalytic site and anion binding exosite with relatively lower affinity but display a K_D in the pM range for the whole enzyme. Multiple binding sites may also explain why collagen binding to immobilized aegyptin is characteristically tight (Figure 2B).

The identification of vWF binding site in collagen as target for aegyptin is particularly relevant taking into account the relative contribution of vWF in the initiation of platelet adhesion and thrombus formation. vWF promotes tethering of platelets to the injury site through binding to both the platelet GPIb and collagen, particularly at high shear rates [3–5]. Accordingly, platelet tethering along the injured vessel wall is reduced by $\approx 80\%$ in mice deficient in vWF; moreover, mutants of vWF with impaired binding to collagen have delayed thrombus formation *in vivo* [40,41]. Likewise, deficiency of GPIb produces a remarkable antithrombotic effect [42], and recent studies have shown that inhibition of GPIb with antibodies profoundly protects mice from ischemic stroke without increasing the risk of intracranial hemorrhage [43]. Altogether, targeting the vWF-binding domain, in addition to GPVI and integrin $\alpha_2\beta_1$ binding sites in collagen appears to be an effective strategy developed by a mosquito salivary gland protein to prevent platelet aggregation.

Aegyptin displays effective anti-thrombotic activity *in vivo*, as indicated by experiments using a laser-induced carotid artery injury in the presence of Rose Bengal, a model where collagen exposure contributes to thrombus formation [24]. Notably, major bleeding was consistently not observable following aegyptin treatment. Examination of additional models will clarify whether the effects of aegyptin *in vivo* is related to blockade of vWF-binding to collagen only, or inhibition of platelet adhesion/activation via integrin $\alpha_2\beta_1$ and/or GPVI. Nevertheless, the findings that aegyptin blocks collagen interaction with different platelet receptors has important implications since it has become clear that integrin $\alpha_2\beta_1$ and GPVI synergistically mediate platelet adhesion and aggregation [7–10]; it is also particularly relevant *vis-à-vis* the relative participation of GPVI in thrombus formation depending on the experimental model employed [44–48]. Therefore, blockade of GPVI-collagen interaction appears to be a useful approach to generate anti-thrombotics without changing the expression levels of GPVI [3].

In an attempt to identify a binding domain responsible for the activity of aegyptin, a series of fragments have been engineered based on the repetitive sequence GEEDA, the pattern of cysteines, and the characteristics of the N- and C-terminus of the inhibitor. Our results demonstrate that the fragment C-terminus-2 of aegyptin (without GEEDA repeats) was relatively effective for binding to collagen and to attenuate platelet aggregation while N-terminus, mid-domain and C-terminus-1 fragments were not. Thus, our findings suggest that the GEEDA motif does not interact with collagen when tested alone, but it cannot be excluded that this domain is active in the intact molecule and contribute at least in part for binding. Finally, it is plausible to envisage aegyptin as a tool to study collagen physiology or as a prototype to develop inhibitors of collagen interaction with ligands [49–51] potentially involved in distinct pathological conditions [11,12].

Methods

Materials

Horse tendon insoluble Horm fibrillar collagen (quaternary, polymeric structure) composed of collagen types I (95%) and III (5%) was from Chrono-Log Corp. (Haverstown, PA). Soluble (tertiary, triple helical) collagens types I and III were from BD Biosciences (Franklin Lakes, NJ). Molecular biology reagents were from Invitrogen (Carlsbad, CA).

Anti-6-His monoclonal antibody was purchased from Covance Co. (Philadelphia, PA). Calcein-AM was from EMD (San Diego, CA). Convulxin was purified as described [23].

Expression of aegyptin domains in mammalian expression system

Aegyptin purification, cloning and expression have been described in detail before [15]. PCR fragments coding for the different domains of aegyptin were amplified (Platinum Supermix, Invitrogen) from a plasmid construct containing the full length aegyptin cDNA. Domain-specific primers were as follows: N-term: (for 5'-AGGCCCATGCCCGAAGATGAAG-3'; rev 5'-TTAATCGGCCGGATCGTTCTTTTCACTACCTTTACTGTCTTC-3'); Aegyptin C-term-1: (for 5'-AGACAGGTGGTTGCATTACTAGAC-3'; rev 5'-TTAGTGGTGGTGGTGGTGGTGACGTCCTTTGGATGAAAC-3'); C-term-2: (for 5'-GGAGGTGACGAAGGAGAAGATAACGC-3'; rev 5'-TTAATCGGCCGGATCGTTCTTTTCACTACCTTTACTGTCTTC-3') Mid-domain: (for 5'-GGACATGACGATGCTGGTGAGG-3'; rev 5'-TTAGTGGTGGTGGTGGTGGTGAAGCATCCTTGAATCTTGG-3'). The reversed primers were designed to have a 6xHis tag followed by a stop codon. PCR-amplified products were gel excised, purified (Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and cloned into VR2001-TOPO vector (modified version of the VR1020 vector, Vical Inc, San Diego, CA) and their sequence and orientation verified by DNA sequencing (DTCS Quick Start Kit, Beckman Coulter). Recombinant protein expression and purification was carried out as before [15].

Dynamic light scattering plot

The purity, identity, and solution state of the purified aegyptin was analyzed using analytical size exclusion chromatography with on-line multi-angle light scattering (SEC-MALS-QELS-HPLC), refractive index (RI) and ultraviolet (UV) detection. The instrument was used as directed by the manufacturer, Waters Corporation (Milford, MA) HPLC (model 2695) and photodiode array (PDA) detector (model 2996) operated by Waters Corporation Empower™ software connected in series to a Wyatt Technology (Santa Barbara, CA) Dawn EOS Light Scattering Detector and Optilab DSP refractive index detector. Wyatt Technology's Astra V software suite was used for data analysis and processing. For separation, a Tosoh Biosciences TSK gel G3000PWxl column (7.8 mm × 30 cm, 6 μm particle size) was used with a TSK gel Guard PWxl column (6.0 mm × 4.0 cm, 12 μm particle size). The column was equilibrated in mobile phase (1.04 mM KH₂PO₄, 2.97 mM Na₂HPO₄ · 7H₂O, 308 mM NaCl, 0.5 M urea, pH 7.4, 0.02% sodium azide) for at least 60 min at 0.5 ml/min prior to sample injection. SEC-MALS-HPLC analysis was performed on the aegyptin using an isocratic elution at 0.5 ml/min in mobile phase. Bio-Rad (Hercules, CA) Gel Filtration Standards were run for size comparisons.

Circular dichroism (CD) of aegyptin

Solutions of aegyptin were dialyzed against PBS and the concentration adjusted to 3 μM. CD spectra were measured by a Jasco J-715 spectropolarimeter with the solutions in a 0.1-cm path length quartz cuvette in a cell holder thermostated by a Neslab RTE-111 circulating water bath. Spectra were scanned four times, from 260 to 190 nm and averaged (speed 50 nm/min, time constant 1s). Spectra were obtained at 25°C. After baseline correction the mean residue ellipticity values were converted using the formula:

$$[\theta] = (10 \times \text{mdeg} \times \text{MRW}) / l \cdot c \cdot 100$$

where m is the measured ellipticity, MRW the mean residue weight, l the pathlength (cm) and c the protein concentration (mg/mL).

Synthesis of collagen-related peptides

Collagen-related peptides (GPO)₁₀, GCO-(GPO)₁₀-GCOG-NH₂ [20] that recognizes collagen binding site for GPVI, and the GFOGER peptide, GPC(GPP)₅GFOGER(GPP)₅GPC [21] that recognizes the integrin $\alpha_2\beta_1$ binding site were synthesized by Synbiosci Co. (Livermore, CA). The RGQOGVMGF peptide, GPC-(GPP)₅-GPOGPSGPRGQOGVMGFQGPCKGNDGAO-(GPP)₅-GPC-NH₂ [22] that recognizes the vWF binding site in collagen was synthesized by Biosynthesis, Inc. (Lewisville, TX). RGQOGVMGF peptide was also obtained without hydroxyproline and referred as RGQPGVMGF peptide. For some control experiments RGQPGVMGF peptide was scrambled (<http://users.umassmed.edu>) and the resulting peptide PGGPDGGF(P)₁₀GPGGKPPNGQGPPSPPGPAGGPGPMPPGPPGGVPGCGGPRPPC-NH₂ synthesized by Biosynthesis Inc (Lewisville, TX)(Supplemental data, Figure S3E). All peptides were purified by HPLC and the molecular mass estimated by mass spectrometry: (GPO)₁₀ (mass spectrum, 3294.7 da; theoretical, 3293.6 da); GFOGER (mass spectrum, 3705.3 da; theoretical, 3704.2 da); RGQOGVMGF (mass spectrum, 5573.2 da; theoretical, 5571.27 da) and scrambled RGQPGVMGF (mass spectrum, 5511.36; theoretical 5511.3 da). For cross-linking, the peptides were re-suspended in PBS and incubated at 4°C for 48 hours, or incubated with SPDP (N-succinimidyl-3-[2-Pyridyldithiol] propionate) reagent from Pierce Co. (Rockford, IL), as described [20]. Control experiments show that RGQOGVMGF supports vWF binding (Figure 3F), (GPO)₁₀ induces platelet aggregation (Figure 4C), and GFOGER supports platelet adhesion in a Ca²⁺-dependent manner (Figure 4D), indicating that all peptides were biologically active according to appropriate *in vitro* assays.

Surface plasmon resonance (SPR) analysis

All SPR experiments were carried out in a T100 instrument (Biacore Inc., Uppsala, Sweden) following the manufacturer's instructions. The Biacore T100 evaluation software was utilized for kinetic analysis. 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 I (30 µg/ml) in acetate buffer pH 4.5 was immobilized over a CM5 sensor via amine coupling, resulting in a final immobilization of 620.8 RU or 1760.2 RU. Peptides were immobilized over a CM5 sensor via amine coupling as recommended by Biacore. The final immobilized levels were as follows: (GPO)₁₀, 662.4 RU; GFOGER, 572.1 RU and RGQOGVMGF, 534.4 RU. In some experiments, soluble collagen I was heat-denatured for 90 min at 98°C in a thermocycler and immobilized at 1871.7 RU. In other experiments, scrambled non-cross-linked RGQPGVMGF peptide (50 µg/ml), soluble collagen III (30 µg/ml) and non cross-linked RGQPGVMGF peptide (50 µg/ml) in acetate buffer pH 4.5 were immobilized in flow cell of the same CM5 sensor chips at respective levels of 1205.8 RU, 719.1 RU and 772.2 RU. 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 µl/min at 25°C. Aegyptin-collagen and aegyptin-peptide complex dissociation was monitored for 1200 s, and the sensor surface was regenerated by a pulse of 20 s of 10 mM HCl at 40 µl/minute. After subtraction of the contribution of bulk refractive index and nonspecific interactions with the CM5 chip surface, the individual association (k_a) and dissociation (k_d) rate constants were obtained by global fitting of data using the two-state reaction (conformational change) interaction model using BIAevaluation™ (Biacore, Inc.) [52]:

Values were then used to calculate the dissociation constant (K_D).

$$K_D = k_{d1} \cdot k_{d2} / k_{a1} (k_{d2} + k_{d1})$$

The values of average squared residual obtained were not significantly improved by fitting data to models that assumed other interactions. Conditions were chosen so that the contribution of mass transport to the observed values of K_D was negligible. In addition, the models in the T100 evaluation software fit for mass transfer coefficient to mathematically extrapolate the true k_a and k_d . Individual sensorgram generated by BIAcore T100 for all interactions described herein are depicted in the Supplemental data (Figures S3A-S3E, S3G, S4A and S4B).

Platelet preparation and aggregation assays

This was performed as described [15]. In some experiments, platelets were labeled with calcein-AM (2 μM , 30 min) and resuspended in Tyrode's buffer (5 mM Hepes, 137 mM NaCl, 27 mM KCl, 12 mM NaHCO_3 , 0.42 mM NaH_2PO_4 , 1 mM MgCl_2 , 5.55 mM glucose, and 0.25% BSA, pH 7.4).

vWf binding to RGQOGVMGF peptide

Polystyrene plates were coated with 100 μl of collagen type III, RGQOGVMGF peptide (30 $\mu\text{g/ml}$) or a 2% (w/v) solution of bovine serum albumin (BSA) diluted in PBS for 2 hours at 37°C. After washing twice with PBS to remove unbound protein, residual binding sites were blocked by adding 5 mg/ml denatured BSA overnight at 4°C. After washing 3 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 0.05 to 3 μM) was added to the well and incubated at 37°C for 1 hour. Wells were washed again and incubated with 3 nM of vWF factor VIII-free (Haematologic Technologies Inc) in TBS-T supplemented with 2% (w/v) BSA. After 1 hour at 37°C, wells were washed 3 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 hour at 37°C. After 3 washes with TBS-T, alkaline phosphatase conjugate anti-rabbit IgG (whole molecule; Sigma) was added (1:10000) and incubated at 37°C for 45 minutes. Before adding the stabilized *p*-nitrophenyl phosphate liquid substrate (Sigma), wells were washed 6 times with TBS-T. After 30 minutes of substrate conversion, the reaction was stopped with 3 N NaOH and absorbance 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.

Aegyptin binding to collagen by solid-phase binding assay

Soluble collagen I (50 μl , 25 $\mu\text{g/ml}$, in PBS, pH 7.4) was immobilized overnight at 4°C. Wells were washed with PBS and blocked with BSA (2% v/v, in PBS) for 2 hours. Then, aegyptin (0–1 μM), diluted in PBS-Tween (PBS, 1% BSA, 0.05% tween) was added. After 2 hours wells were washed in PBS-Tween and incubated with anti-his antibody (1 $\mu\text{g/ml}$) in the same buffer. After 1 hour, wells were washed and incubated with alkaline phosphatase-coupled anti-mouse antibody (1:3000, in PBS-Tween) for 1 hour. Before adding the stabilized *p*-nitrophenyl phosphate liquid substrate (Sigma), wells were washed 4 times with

PBS-Tween. Colorimetric analysis was performed by measuring the absorbance values at 405 nm. The (apparent) K_d values for aegyptin-collagen interaction were calculated by nonlinear regression analysis of the binding data (GraphPad software, USA). Assays were performed in quintuplicates.

Binding of aegyptin-FITC to fibrillar collagen

The fluorescein-EX dye (Molecular Probes) was utilized for labeling of approximately 250 μ g of recombinant aegyptin, following the manufacture's recommendation. Coverslips (22×22 mm, no. 1.5) were treated with H_2SO_4 : H_2O_2 (4:1) for 20 minutes to remove contaminants, followed by ultrasonic washing with deionized water and ultraviolet cleaning. Coverslips were coated with fibrillar collagen (100 μ g/ml; Chronolog-Par) for 10 minutes, rinsed in deionized water, and incubated for 30 min with denaturated BSA (7 mg/ml). Coverslips were treated with 100 μ l of aegyptin-FITC (0.1 μ M) for 15 minutes, and inhibitor was removed by inverting and touching the borders of coverslips with precision wipes (Kimberly-Clark, Ontario, Canada) and mounted for imaging. Differential interference contrast (DIC) and fluorescent (488nm) images were obtained with a Leica DMI6000 microscope (Leica Microsystems, Inc., Bannockburn, IL) using 100 $\times$ objective with 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).

Platelet adhesion assay under static conditions

Inhibition of platelet adhesion to immobilized collagen or integrin related peptide was examined by fluorometry. Microfluor black microtiter 96-well plate (ThermoLabsystems, Franklin, MA, USA) were coated with 1 μ g of fibrillar Horm collagen or 5 μ g of GFOGER overnight at 4°C in PBS pH 7.2. Wells were washed twice with TBS and then incubated with 2% BSA in Tyrode buffer to block nonspecific binding sites. After 1h the plate was washed twice with Tyrode buffer. Different concentrations of recombinant aegyptin in Tyrode buffer were transferred into wells and incubated for 1h at room temperature. Wells were washed three times with Tyrode buffer and 50 μ l calcein-AM labeled platelets were transferred to the well and incubated for 1.5 h at room temperature. After six washes with Tyrode buffer the platelet adhesion was estimated by measuring the fluorescence of the associated cells to the wells using a SpectraMax GeminiXPS fluorimeter (Molecular Devices, Sunnyvale, CA, USA) with 490/520-nm (excitation/emission) filters.

Animals

Adult Wistar rats (males) weighting 200–250 g were housed under controlled conditions of temperature ($24 \pm 1^\circ\text{C}$) and light (12 h light starting at 07:00 am), and all experiments were conducted in accordance with standards of animal care defined by the Institutional Committee.

Photochemically induced carotid artery thrombosis in rats

Rats were anesthetized with intramuscular xylazin (16 mg/kg) followed by ketamine (100 mg/kg). The right common carotid artery was isolated through a midline cervical incision, and the blood flow was continuously monitored using a 1PRB Doppler flow probe coupled to a TS420 flowmeter (Transonic Systems, Ithaca, NY) as described [25]. Fifteen minutes before induction of thrombosis, animals were injected in the cava vein with aegyptin (50 or 100 μ g/kg) or PBS (control). Thrombosis was induced by slow injection (over 2 min) of 90 mg/kg body weight of rose bengal dye (Fisher Scientific, Pittsburgh, PA) into the cava vein at a concentration of 60 mg/ml. Just before injection, a 1.5 mW, 540 nm green light laser (Melles Griot, Carlsbad, CA) was applied to the desired site of injury from a distance of 3

cm. Mean carotid artery blood flow was monitored for 80 min or until stable occlusion occurred, at which time the experiment was terminated. Fifteen minutes before the injection of rose bengal dye, some animals were injected with aegyptin at dose of 50 µg/Kg or 100 µg/kg. Stable occlusion was defined as a blood flow of 0 ml/min for ≥ 10 min.

Bleeding

Wistar rats (both sex, ~100 g body weight) were anesthetized as above with a combination of xylazine and ketamine (16 and 100 mg/kg, respectively). A cannula was inserted into the right carotid artery for administration of different doses of aegyptin or heparin. After 15 min, the tail was cut within 2 mm of diameter and carefully immersed in 40 ml distilled water at room temperature. The hemoglobin content in water solution (absorbance at 540 nm) was used to evaluate blood loss [53]. Appropriate controls (*i.v.* injection of PBS) were run in parallel.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed with one-way ANOVA, followed by a post-hoc test (Dunnett) using the statistical package GraphPad Prism 4.0 (GraphPad software, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Khoshnoodi J, Cartailier JP, Alvares K, Veis A, Hudson BG. Molecular recognition in the assembly of collagens: terminal noncollagenous domains are key recognition modules in the formation of triple helical protomers. *J Biol Chem.* 2006; 281:38117–38121. [PubMed: 17082192]
2. Heino J. The collagen family members as cell adhesion proteins. *Bioessays.* 2007; 29:1001–1010. [PubMed: 17876790]
3. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? *Blood.* 2003; 102:449–461. [PubMed: 12649139]
4. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell.* 1998; 94:657–666. [PubMed: 9741630]
5. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest.* 2005; 115:3355–3362. [PubMed: 16322780]
6. Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, Le Couedic JP, Goodearl A, Dussault B, Fraser C, Vainchenker W, Villeval JL. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood.* 2000; 96:1798–1807. [PubMed: 10961879]
7. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin α IIb β 3 signaling in platelets. *J Thromb Haemost.* 2005; 3:1752–1762. [PubMed: 16102042]
8. Gibbins JM. Platelet adhesion signalling and the regulation of thrombus formation. *J Cell Sci.* 2004; 117:3415–3425. [PubMed: 15252124]

9. Jung SM, Moroi M. Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin $\alpha_2\beta_1$. *J Biol Chem*. 2000; 275:8016–8026. [PubMed: 10713121]
10. Chen H, Kahn ML. Reciprocal signaling by integrin and nonintegrin receptors during collagen activation of platelets. *Mol Cell Biol*. 2003; 23:4764–4777. [PubMed: 12832464]
11. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007; 357:2482–2494. [PubMed: 18077812]
12. Spiel AO, Gilbert JC, Jilma B. von Willebrand factor in cardiovascular disease: focus on acute coronary syndromes. *Circulation*. 2008; 117:1449–1459. [PubMed: 18347221]
13. Fox JW, Serrano SM. Approaching the golden age of natural product pharmaceuticals from venom libraries: an overview of toxins and toxin-derivatives currently involved in therapeutic or diagnostic applications. *Curr Pharm Des*. 2007; 13:2927–2934. [PubMed: 17979737]
14. Ribeiro JM, Francischetti IM. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol*. 2003; 48:73–88. [PubMed: 12194906]
15. Calvo E, Tokumasu F, Marinotti O, Villeval JL, Ribeiro JM, Francischetti IM. 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. *J Biol Chem*. 2007; 282:26928–26938. [PubMed: 17650501]
16. Yoshida S, Sudo T, Niimi M, Tao L, Sun B, Kambayashi J, Watanabe H, Luo E, Matsuoka H. Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito. *Blood*. 2008; 111:2007–2014. [PubMed: 18056842]
17. Calvo E, Andersen J, Francischetti IM, de LCM, deBianchi AG, James AA, Ribeiro JM, Marinotti O. The transcriptome of adult female *Anopheles darlingi* salivary glands. *Insect Mol Biol*. 2004; 13:73–88. [PubMed: 14728669]
18. Francischetti IM, Valenzuela JG, Ribeiro JM. Anophelin: kinetics and mechanism of thrombin inhibition. *Biochemistry (Mosc)*. 1999; 38:16678–16685.
19. van de Locht A, Lamba D, Bauer M, Huber R, Friedrich T, Kroger B, Hoffken W, Bode W. Two heads are better than one: crystal structure of the insect derived double domain Kazal inhibitor rhodniin in complex with thrombin. *EMBO J*. 1995; 14:5149–5157. [PubMed: 7489704]
20. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin $\alpha_2\beta_1$ 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for $\alpha_2\beta_1$ 1-independent platelet reactivity. *Biochem J*. 1995; 306(Pt 2):337–344. [PubMed: 7534064]
21. Raynal N, Hamaia SW, Siljander PR, Maddox B, Peachey AR, Fernandez R, Foley LJ, Slatton DA, Jarvis GE, Farndale RW. Use of synthetic peptides to locate novel integrin $\alpha_2\beta_1$ -binding motifs in human collagen III. *J Biol Chem*. 2006; 281:3821–3831. [PubMed: 16326707]
22. Lisman T, Raynal N, Groeneveld D, Maddox B, Peachey AR, Huizinga EG, de Groot PG, Farndale RW. A single high-affinity binding site for von Willebrand factor in collagen III, identified using synthetic triple-helical peptides. *Blood*. 2006; 108:3753–3756. [PubMed: 16912226]
23. Francischetti IM, Saliou B, Leduc M, Carlini CR, Hatmi M, Randon J, Faily A, Bon C. Convulxin, a potent platelet-aggregating protein from *Crotalus durissus terrificus* venom, specifically binds to platelets. *Toxicon*. 1997; 35:1217–1228. [PubMed: 9278971]
24. Rosen ED, Raymond S, Zollman A, Noria F, Sandoval-Cooper M, Shulman A, Merz JL, Castellino FJ. Laser-induced noninvasive vascular injury models in mice generate platelet- and coagulation-dependent thrombi. *Am J Pathol*. 2001; 158:1613–1622. [PubMed: 11337359]
25. Melo FR, Mourao PA. An algal sulfated galactan has an unusual dual effect on venous thrombosis due to activation of factor XII and inhibition of the coagulation proteases. *Thromb Haemost*. 2008; 99:531–538. [PubMed: 18327401]
26. Penz S, Reininger AJ, Brandl R, Goyal P, Rabie T, Bernlochner I, Rother E, Goetz C, Engelmann B, Smethurst PA, Ouwehand WH, Farndale R, Nieswandt B, Siess W. Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. *FASEB J*. 2005; 19:898–909. [PubMed: 15923400]
27. Toschi V, Gallo R, Lettino M, Fallon JT, Gertz SD, Fernandez-Ortiz A, Chesebro JH, Badimon L, Nemerson Y, Fuster V, Badimon JJ. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation*. 1997; 95:594–599. [PubMed: 9024145]

28. Francischetti IM, Seydel KB, Monteiro RQ. Blood coagulation, inflammation, and malaria. *Microcirculation*. 2008; 15:81–107. [PubMed: 18260002]
29. Giudici C, Raynal N, Wiedemann H, Cabral WA, Marini JC, Timpl R, Bachinger HP, Farndale RW, Sasaki T, Tenni R. Mapping of SPARC/BM-40/Osteonectin-binding Sites on Fibrillar Collagens. *J Biol Chem*. 2008; 283:19551–19560. [PubMed: 18487610]
30. Konitsiotis AD, Raynal N, Bihan D, Hohenester E, Farndale RW, Leitingner B. Characterization of high affinity binding motifs for the discoidin domain receptor DDR2 in collagen. *J Biol Chem*. 2008; 283:6861–6868. [PubMed: 18201965]
31. Harsfalvi J, Stassen JM, Hoylaerts MF, Van Houtte E, Sawyer RT, Vermeylen J, Deckmyn H. Calin from *Hirudo medicinalis*, an inhibitor of von Willebrand factor binding to collagen under static and flow conditions. *Blood*. 1995; 85:705–711. [PubMed: 7833474]
32. Connolly TM, Jacobs JW, Condra C. An inhibitor of collagen-stimulated platelet activation from the salivary glands of the *Haementeria officinalis* leech. I. Identification, isolation, and characterization. *J Biol Chem*. 1992; 267:6893–6898. [PubMed: 1551897]
33. Barnes CS, Krafft B, Frech M, Hofmann UR, Papendieck A, Dahlems U, Gellissen G, Hoylaerts MF. Production and characterization of saratin, an inhibitor of von Willebrand factor-dependent platelet adhesion to collagen. *Semin Thromb Hemost*. 2001; 27:337–348. [PubMed: 11547356]
34. White TC, Berny MA, Robinson DK, Yin H, DeGrado WF, Hanson SR, McCarty OJ. The leech product saratin is a potent inhibitor of platelet integrin $\alpha 2\beta 1$ and von Willebrand factor binding to collagen. *FEBS J*. 2007; 274:1481–1491. [PubMed: 17489103]
35. Lasser G, Guchhait P, Ellsworth JL, Sheppard P, Lewis K, Bishop P, Cruz MA, Lopez JA, Fruebis J. C1qTNF-related protein-1 (CTRP-1): a vascular wall protein that inhibits collagen-induced platelet aggregation by blocking VWF binding to collagen. *Blood*. 2006; 107:423–430. [PubMed: 16195328]
36. Serrano SM, Jia LG, Wang D, Shannon JD, Fox JW. Function of the cysteine-rich domain of the haemorrhagic metalloproteinase atrolysin A: targeting adhesion proteins collagen I and von Willebrand factor. *Biochem J*. 2005; 391:69–76. [PubMed: 15929722]
37. Somasundaram R, Schuppan D. Type I, II, III, IV, V, and VI collagens serve as extracellular ligands for the isoforms of platelet-derived growth factor (AA, BB, and AB). *J Biol Chem*. 1996; 271:26884–26891. [PubMed: 8900172]
38. Leikina E, Merts MV, Kuznetsova N, Leikin S. Type I collagen is thermally unstable at body temperature. *Proc Natl Acad Sci U S A*. 2002; 99:1314–1318. [PubMed: 11805290]
39. Egles C, Shamis Y, Mauney JR, Volloch V, Kaplan DL, Garlick JA. Denatured collagen modulates the phenotype of normal and wounded human skin equivalents. *J Invest Dermatol*. 2008; 128:1830–1837. [PubMed: 18200055]
40. Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO, Wagner DD. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proc Natl Acad Sci U S A*. 1998; 95:9524–9529. [PubMed: 9689113]
41. Marx I, Christophe OD, Lenting PJ, Rupin A, Vallez MO, Verbeuren TJ, Denis CV. Altered thrombus formation in von Willebrand factor-deficient mice expressing von Willebrand factor variants with defective binding to collagen or GPIIb/IIIa. *Blood*. 2008; 112:603–609. [PubMed: 18487513]
42. Konstantinides S, Ware J, Marchese P, Almus-Jacobs F, Loskutoff DJ, Ruggeri ZM. Distinct antithrombotic consequences of platelet glycoprotein Ib α and VI deficiency in a mouse model of arterial thrombosis. *J Thromb Haemost*. 2006; 4:2014–2021. [PubMed: 16961609]
43. Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B, Stoll G. Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. *Circulation*. 2007; 115:2323–2330. [PubMed: 17438148]
44. Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC. Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. *Blood*. 2006; 107:3902–3906. [PubMed: 16455953]

45. Mangin P, Yap CL, Nonne C, Sturgeon SA, Goncalves I, Yuan Y, Schoenwaelder SM, Wright CE, Lanza F, Jackson SP. Thrombin overcomes the thrombosis defect associated with platelet GPVI/FcRgamma deficiency. *Blood*. 2006; 107:4346–4353. [PubMed: 16391010]
46. Kalia N, Auger JM, Atkinson B, Watson SP. Critical role of FcR gamma-chain, LAT, PLCgamma2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury in vivo. *Microcirculation*. 2008; 15:325–335. [PubMed: 18464161]
47. Munnix IC, Strehl A, Kuijpers MJ, Auger JM, van der Meijden PE, van Zandvoort MA, oude Egbrink MG, Nieswandt B, Heemskerk JW. The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. *Arterioscler Thromb Vasc Biol*. 2005; 25:2673–2678. [PubMed: 16254207]
48. Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohnhofer D, Heinzmann U, Nieswandt B. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med*. 2003; 197:41–49. [PubMed: 12515812]
49. Muzard J, Sarda-Mantel L, Loyau S, Meulemans A, Louedec L, Bantsimba-Malanda C, Hervatin F, Marchal-Somme J, Michel JB, Le Guludec D, Billiald P, Jandrot-Perrus M. Non-invasive molecular imaging of fibrosis using a collagen-targeted peptidomimetic of the platelet collagen receptor glycoprotein VI. *PLoS One*. 2009; 4:e5585. [PubMed: 19440310]
50. Cabral WA, Makareeva E, Letocha AD, Scribanu N, Fertala A, Steplewski A, Keene DR, Persikov AV, Leikin S, Marini JC. Y-position cysteine substitution in type I collagen (alpha1(I) R888C/p.R1066C) is associated with osteogenesis imperfecta/Ehlers-Danlos syndrome phenotype. *Hum Mutat*. 2007; 28:396–405. [PubMed: 17206620]
51. Vanhoorelbeke K, Ulrichs H, Schoolmeester A, Deckmyn H. Inhibition of platelet adhesion to collagen as a new target for antithrombotic drugs. *Curr Drug Targets Cardiovasc Haematol Disord*. 2003; 3:125–140. [PubMed: 12769639]
52. Morton TA, Myszka DG, Chaiken IM. Interpreting complex binding kinetics from optical biosensors: a comparison of analysis by linearization, the integrated rate equation, and numerical integration. *Anal Biochem*. 1995; 227:176–185. [PubMed: 7668379]
53. Herbert JM, Herault JP, Bernat A, van Amsterdam RG, Vogel GM, Lormeau JC, Petitou M, Meuleman DG. Biochemical and pharmacological properties of SANORG 32701. Comparison with the "synthetic pentasaccharide" (SR 90107/ORG 31540) and standard heparin. *Circ Res*. 1996; 79:590–600. [PubMed: 8781492]

Fig. 1. Biophysical properties of aegyptin

(A) Chromatographic analysis of aegyptin by size exclusion TSK gel G3000PW (arrow, apparent molecular mass 110 kDa) is superimposed to the elution pattern of molecular mass markers (in blue). *Inset* shows SDS-PAGE of purified recombinant aegyptin (arrowhead). Molecular mass standards used: thyroglobulin (670 kDa), immunoglobulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.4 kDa). (B) Inline multi-angle light scatter. The solid and blue lines represent absorbance 280 nm and MALS results, respectively. *Inset* shows the results between 10–20 mls. (C) CD spectra of aegyptin. *Inset* shows the proportion of α -helix, β -sheet, β -turn and unordered structures.

Fig. 2. Aegyptin interaction with collagen

(A) Surface plasmon resonance. Sensorgrams in black lines show aegyptin (in nM: *a*, 20; *b*, 10; *c*, 5; *d*, 2.5; *e*, 1.25) binding to immobilized soluble collagen type I. Data fitting using global two-state binding model is displayed as red line. (B) Sensorgrams show collagen (in nM: *a*, 5; *b*, 2.5; *c*, 1.25; *d*, 0.625; *e*, 0.3; *f*, 0.15 and *g*, 0.075) binding to immobilized aegyptin. (C) Solid phase binding assay. Aegyptin (0–1 μ M) was incubated with immobilized collagen and binding estimated with anti-his mouse monoclonal antibody as described in Methods. (D) Fluorescence microscopy. Cover slips coated with fibrillar collagen were incubated with aegyptin-FITC for 20 min at room temperature and analyzed under fluorescence microscope (right upper panel), as described in Methods. Collagen incubated with PBS (negative control) did not display auto fluorescence under the same conditions (right lower panel). DIC for each condition is shown in the left lower and upper panels.

Fig. 3. Aegyptin displays high affinity for vWF binding site of collagen

Sensorgrams show aegyptin binding to immobilized (A) cross-linked RGQOGVMGF, (B) linear RGQOGVMGF, (C) cross-linked hydroxyproline-less RGQPGVMGF, (D) linear hydroxyproline-less RGQPGVMGF, and (G) heat-denatured collagen (90 min at 98°C). In (E) aegyptin was injected in different flow cells of the same sensor chip containing immobilized scrambled RGQPGVMGF, or collagen type III or RGQPGVMGF. Concentrations of recombinant aegyptin for (A)–(D) were (in nM: a, 50; b, 25; c, 12.5; d, 6.75; e, 3.1), for (E) was 1 μ M; and for (G) was (in nM: a, 150; b, 75; c, 37.5; d, 18; e, 9; f, 4.5). Dissociation of aegyptin-ligand complex was monitored for 1800 seconds, and a global two-state reaction model was used to calculate kinetic parameters. (F) Inhibition of vWF

binding to cross-linked RGQOGVMGF was estimated by ELISA in the presence of indicated concentrations of aegyptin.

Fig. 4. Aegyptin displays low affinity for GPVI or integrin $\alpha 2\beta 1$ binding sites of collagen
 Sensorgrams shows aegyptin binding to immobilized (A) cross-linked (GPO)₁₀ or (B) cross-linked GFOGER. Aegyptin concentration for (A) was (in μM : a, 2; b, 1.5; c, 1; d, 0.75; e, 0.5; and f, 0.25) and for (B) was (in μM : a, 3; b, 2; c, 1; d, 0.5; e, 0.3; and f, 0.15). Dissociation of aegyptin-ligand complex was monitored for 1800 seconds, and a global two-state reaction model was used to calculate kinetic parameters. (C) Functional assay using human platelet-rich plasma shows that aegyptin is ineffective to inhibit platelet responses to (GPO)₁₀ (2.5 $\mu\text{g/ml}$) but prevents collagen (2 $\mu\text{g/ml}$)-induced platelet aggregation. (D) Aegyptin failed to prevent washed human platelet adhesion to GFOGER under static conditions, but effectively inhibits platelet adhesion to collagen. No adhesion was detectable in the presence of EDTA.

Fig. 5. C-terminal-2 fragment of aegyptin binds to collagen

(A) Diagram of the constructs used for cloning and expression. (B) SPR experiments show C-terminus 2 fragment binding to aegyptin. (C) Sensorgrams of C-terminus-2 fragment (in nM: *a*, 250; *b*, 120; *c*, 60; *d*, 30; *e*, 15 and *f*, 5) binding to immobilized soluble collagen type I. Dissociation of aegyptin-collagen complex was monitored for 1800 seconds, and a global two-state binding model was used to calculate kinetic parameters. (D) Human platelet-rich plasma ($2 \times 10^5/\mu\text{l}$) was incubated with C-terminus-2 fragment (in μM : *a*, 0; *b*, 3; and *c*, 10) for 1 minute followed by addition of fibrillar Horm collagen ($2 \mu\text{g}/\text{ml}$, final concentration). Platelet aggregation was estimated by turbidimetry under test-tube stirring conditions. The tracings represent a typical experiment.

Fig. 6. Aegyptin prevents thrombus formation *in vivo*

(A) aegyptin (50 or 100 $\mu\text{g/kg}$) or PBS (control) was injected in the cava vein of rats and thrombosis was induced by slow injection (over 2 min) of 90 mg/kg body weight of Rose Bengal dye into the cava vein at a concentration of 60 mg/mL. Before injection, green light laser was applied to the desired site of injury from a distance of 3 cm and remained on for 80 minutes or until stable occlusion occurred. The number of animals tested for each condition is shown in the Figure. (B) Determination of the bleeding. Aegyptin at the indicated doses was administered *i.v.*; after 15 min of administration, the rat tail was cut 2 mm from the tip. The tail was carefully immersed in 40 ml of distilled water at room temperature, and blood loss (hemoglobin content) was estimated at 540 nm, after 60 min, as compared to a standard

curve. Animals that received PBS were taken as control. In some experiments animals received heparin (1 mg/kg). Data represent the mean $\pm$ SEM of results obtained with 7 to 10 animals. *, $P < 0.05$.

Kinetics of aegyptin interaction with soluble collagen type I, immobilized in the CM5 sensor chips at 620 and 1760 RU. Data were fitted using two different equations and results reported below. Responses were obtained by injecting recombinant aegyptin over immobilized collagen for 180 s and dissociation for 2000 s, at a flow rate of 30 μ l/min. Experiments were performed in triplicates.

Table 1

	$ka1$ ($M^{-1}s^{-1}$)	$kd1$ (s^{-1})	$ka2$ (s^{-1})	$kd2$ (s^{-1})	K_D	χ^2
<i>Langmuir (1:1 binding)</i>						
Collagen type I (620 RU)	9.78×10^8	4.52	---	---	4.71 nM	1.44
Collagen type I (1760 RU)	1.14×10^9	5.60	---	---	4.94 nM	8.13
<i>Two-state reaction (Conformation change)</i>						
Collagen type I (620 RU)	2.77×10^6	2.74×10^{-2}	1.09×10^{-3}	1.62×10^{-3}	5.92 nM	0.143
Collagen type I (1760 RU)	4.20×10^7	3.40×10^{-1}	3.21×10^{-4}	1.01×10^{-3}	6.05 nM	0.744

Kinetics of aegyptin interaction with soluble collagen type I, collagen peptides and heat-denatured collagen. Responses were obtained by injecting recombinant aegyptin over immobilized peptides and proteins for 180 s and dissociation for 1200 s, at a flow rate of 30 μ l/min. Data were fitted using two-state reaction model. Cross-L; cross-linked peptides; Linear; non-cross-linked peptides.

Table 2

	$ka1$ ($M^{-1}s^{-1}$)	$kd1$ (s^{-1})	$ka2$ (s^{-1})	$kd2$ (s^{-1})	K_D	χ^2
Collagen type I	2.770×10^6	0.02740	0.001090	0.001620	5.92 nM	0.143
(RGQGVVMGF)-Cross-L	3.237×10^5	0.01598	0.001371	0.001294	23.98 nM	0.775
(RGQGVVMGF)-Linear	3.266×10^5	0.01732	0.000688	0.002559	41.81 nM	1.21
(RGQGVVMGF)-Cross-L	3.734×10^5	0.00438	0.002920	0.004704	7.24 nM	4.19
(RGQGVVMGF)-Linear	4.261×10^5	0.00329	0.001160	0.002506	5.26 nM	1.79
Collagen denatured	6.742×10^5	0.01876	0.002062	0.000274	3.32 nM	2.75
(GPO) ₁₀ -Cross-L	1.120×10^5	1.25360	0.005891	0.040440	9.76 μM	0.422
(GFOGER)-Cross-L	4.058×10^5	0.94320	0.000366	0.002613	2.40 μM	0.803