Novel Family of Insect Salivary Inhibitors Blocks Contact Pathway Activation by Binding to Polyphosphate, Heparin, and Dextran Sulfate

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Objective—Polyphosphate and heparin are anionic polymers released by activated mast cells and platelets that are known to stimulate the contact pathway of coagulation. These polymers promote both the autoactivation of factor XII and the assembly of complexes containing factor XI, prekallikrein, and high-molecular-weight kinogen. We are searching for salivary proteins from blood-feeding insects that counteract the effect of procoagulant and proinflammatory factors in the host, including elements of the contact pathway.

Approach and Results—Here, we evaluate the ability of the sand fly salivary proteins, PdSP15a and PdSP15b, to inhibit the contact pathway by disrupting binding of its components to anionic polymers. We attempt to demonstrate binding of the proteins to polyphosphate, heparin, and dextran sulfate. We also evaluate the effect of this binding on contact pathway reactions. We also set out to determine the x-ray crystal structure of PdSP15b and examine the determinants of relevant molecular interactions. Both proteins bind polyphosphate, heparin, and dextran sulfate with high affinity. Through this mechanism they inhibit the autoactivation of factor XII and factor XI, the reciprocal activation of factor XII and prekallikrein, the activation of factor XI by thrombin and factor XIIa, the cleavage of high-molecular-weight kinogen in plasma, and plasma extravasation induced by polyphosphate. The crystal structure of PdSP15b contains an amphipathic helix studded with basic side chains that forms the likely interaction surface.

Conclusions—The results of these studies indicate that the binding of anionic polymers by salivary proteins is used by blood feeders as an antihemostatic/anti-inflammatory mechanism. (Arterioscler Thromb Vasc Biol. 2013;33:2759-2770.)

Key Words: blood coagulation factor inhibitors ■ bradykinin ■ factor XI ■ factor XII ■ inflammation ■ kallikreins ■ leishmania

The contact pathway of coagulation in mammals is initiated by conversion of coagulation factor XII (FXII) to FXIIa in an autocleavage reaction that occurs when the zymogen binds to an anionic surface.1 FXIIa goes on to convert factor XI (FIX) to FXIa, which in turn activates factor IX (FIX), the protease component of the intrinsic factor Xase complex, leading to thrombin production and fibrin deposition.1,2 Although a deficiency in FXII leads to an increase in the clotting time in vitro, no significant bleeding disorder is associated with this.3 More recent studies with rodent models have shown that FXII deficiency protects against thrombosis after vascular injury, and additional studies have shown that inhibitors of this protease effectively reduce the generation and stability of thrombi in vivo, despite the lack of an effect on hemostasis.4,5 In addition to its role in coagulation, FXIIa converts prekallikrein to kallikrein, a serine protease closely related to FXI that is responsible for cleavage of the plasma protein high-molecular-weight kinogen (HK) to give the peptide bradykinin. Kallikrein also amplifies the contact pathway by converting FXII to FXIIa.1 When released from the kininogen, bradykinin causes a rapid increase in vascular permeability, edema, and an immediate sensation of pain.1 The activation of FXI and prekallikrein, as well as the cleavage of HK, is also enhanced in the presence of anionic surfaces, suggesting that surface molecules are an important component of multiple proteolytic cycles.

A variety of negatively charged materials are known to act as surfaces in the auto activation of FXII. Untreated glass efficiently activates the pathway, and earth materials, such as kaolin, are used to initiate coagulation in the activated partial...
thromboplastin time test. Dextran sulfate (DS), a sulfated glycan, has long been used as an in vitro activator of FXII in laboratory studies. Endogenous anionic polymers, including polyP, heparin, and nucleic acids, activate FXII and are considered candidates to serve in this role in vivo. PolyP is released by platelets and mast cells, whereas heparin is released only by mast cells after activation by IgE-antigen complexes. On release, the highly sulfated glycosaminoglycan provides an anionic surface for the activation of FXII and has been shown to activate the contact pathway in vitro and in vivo. The binding of heparin by antithrombin gives it its familiar anticoagulant properties in the circulation, and as a consequence heparin-induced activation of the contact pathway leads only to bradykinin formation and not coagulation. PolyP chains are also potent activators of the contact pathway and have been shown to support the efficient autoactivation of FXII to FXIIa, its mature enzymatic form. Unlike heparin, however, this polymer is a potent endogenous cofactor for activation of FXI by thrombin and does not activate antithrombin. It therefore exhibits both procoagulant and proinflammatory activities in vivo and may be an important component of normal hemostasis.

The saliva of blood-feeding arthropods contains a variety of peptides and small molecules that act to inhibit the hemostatic and inflammatory systems of the host. Proteins and peptides attacking various points in the coagulation cascade have been described, including targets in the contact pathway. Increases in bradykinin and histamine levels resulting from the activation of mast cells during blood feeding produce many of the symptoms associated with an insect bite, including itching, swelling, and pain. To a feeding insect these rapid responses in the skin are a significant impediment to the ingestion of blood. Sensations of pain and itching alert the host to the insect’s presence and may cause it to interrupt feeding, whereas edema may also make the physical process of blood intake more difficult.

Here, we show that the PdSP15 proteins, a group of salivary proteins from the African sand fly Phlebotomous duboscqi, a vector of parasitic Leishmania species, bind negatively charged surfaces, including polyP, heparin, and DS. By competing with FXII for binding sites they inhibit activation of the zymogen and consequently the processes of coagulation and bradykinin production in plasma. We have also determined the x-ray crystal structure of one of these proteins and found it to contain a positively charged surface dominated by a single α-helix studded with the side chains of basic amino acid residues along the length of its solvent-facing sides. This is the likely region for interaction with anionic surfaces.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DS</td>
<td>dextran sulfate</td>
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<tr>
<td>FXI</td>
<td>factor XI</td>
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<tr>
<td>FXII</td>
<td>factor XII</td>
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<tr>
<td>HK</td>
<td>high-molecular-weight kininogen</td>
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<td>polyP</td>
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Results
PdSP15a and b are closely related (86% amino acid identity) members of the insect odorant-binding protein family found in the saliva of P. duboscqi. They are also very similar in sequence to the SP15 protein of the related sand fly P. papatasi (=65% amino acid identity; Figure I in the online-only Data Supplement). Proteomic analysis has shown this group to be the most abundant group of proteins in the saliva of P. duboscqi.

Recombinant PdSP15a and b were tested for inhibition of the clotting of normal plasma in the activated partial thromboplastin time assay, where coagulation is initiated by addition of an anionic colloidal silica suspension (Cephen) and calcium. In the presence of either of the salivary proteins, an ~5-fold delay in clotting time was observed (Figure 1A and 1B). The degree of inhibition increased as a function of inhibitor concentration, up to the maximum tested concentration of 10 μmol/L. Both PdSP15 proteins produced nearly identical levels of inhibition suggesting that they act by the same mechanism. PdSP15a also inhibited coagulation in FIX, factor X, and FXI-deficient plasmas in a concentration-dependent manner (Figure 1D–1F), whereas clotting of FXII-deficient plasma was not inhibited (Figure 1C), suggesting a dependence of inhibition on the presence of FXII. In addition, we found that PdSP15a and b effectively inhibited the reciprocal activation of FXII and prekallikrein in the presence of DS (Figure 1G and 1H) further indicating that PdSP15 proteins prevent coagulation by interfering with the autoactivation or catalytic function of FXII/FXIIa.

We tested whether a block in the autoactivation of FXII was responsible for the results described above. FXII was activated by the addition of DS (1 μg/mL; molecular weight=500,000), polyP (P700; 52.5 μmol/L), or heparin (20 μg/mL) in the presence of 150 nmoL FXII and the chromogenic substrate S-2302. The autoactivation of FXII in the presence of all 3 initiating polymers was reduced substantially in the presence of PdSP15a and b at concentrations of ≥500 nmoL, with essentially complete inhibition being attained at concentrations of ≥1 μmol/L (Figure 2A–2F). The quantity of FXIIa produced in the presence of polyP was reduced ~50% at a concentration of 250 nmoL/L, 90% at 500 nmoL/L, and 99% at 4 μmol/L (Figure 2G). Like FXII, FXI is autoactivated in the presence of DS in vitro although this pathway is not thought to be important in vivo as a means of initiating coagulation. When the DS-mediated activation of FXI was measured continuously as described above with FXII, PdSP15a was found to inhibit the process in a concentration-dependent manner (Figure 2H). Salivary gland homogenates produced similar activity to the recombinant proteins. Activation of FXII by polyP was inhibited ~50% at a concentration of 5.6 ng/μL (ca. 0.6 salivary gland pair per 100 μL reaction) of salivary protein and 90% at a concentration of 15 ng/μL (Figure 2I). If the salivary gland extract was added after the coincubation of polyP and FXII, but before the addition of chromogenic substrate, essentially no inhibition was observed, suggesting that the inhibitor blocks the activation of FXII but not the amidolytic activity of FXIIa (Figure 2I).

The results of autoactivation studies suggested that the PdSP15 proteins block the cleavage of FXII and FXI, either
by binding with the proenzymes themselves or by blocking protease binding sites on anionic polymers. We evaluated PdSP15a as a direct inhibitor of FXIIa, kallikrein, or FXIa by measuring the cleavage of their respective chromogenic substrates in the presence and absence of PdSP15b. In the absence of anionic polymers, PdSP15b does not inhibit substrate cleavage by any of the 3 enzymes at concentrations ≤ 2 μmol/L of inhibitor, suggesting that PdSP15b does not bind these serine proteases or that formation of a complex does not block the access of chromogenic substrate to the catalytic site of the enzyme (Figure 3A–3C).

The activation of FXI by thrombin is greatly enhanced in the presence of polyP, demonstrating that a ternary complex is necessary for optimal activation.⁷ We evaluated the effect of PdSP15a on this process in the presence of 2 μmol/L polyP (P700) or in its absence. In the presence of polyP, PdSP15a potently inhibited FXI activation by FXIIa, with an inhibitory concentration₅₀ value of ≈ 75 nmol/L (Figure 3D). In the absence of polyP, the rate of FXI cleavage by thrombin production was greatly diminished and was not inhibited by PdSP15a at concentrations ≤ 1 μmol/L (Figure 3E). In a similar manner, the activation of FXI by FXIIa is known to be strongly enhanced by DS.²⁷ We found that in the presence of 0.2 μg/mL DS, the activation of FXI by FXIIa was inhibited by PdSP15b with an approximate inhibitory concentration₅₀ value of 250 nmol/L (Figure 3F). In the absence of DS, the rate of FXI activation was reduced and was not inhibited by PdSP15b (Figure 3G). Taken together, these results with DS and polyP suggest that PdSP15a and b act by inhibiting assembly of the thrombin or FXIIa complexes with FXI on the anionic polymer surface rather than by binding with the coagulation factors themselves.

The sequestration of anionic polymer–binding sites suggested that PdSP15 proteins would inhibit polyP-initiated coagulation by preventing autoactivation of FXII and other downstream reactions in plasma. We found that adding polyP (P700) to plasma at a concentration of 52.5 μmol/L produced a reduction in the clotting time of recalciﬁed plasma from a mean of ≈640 s in the absence of polyP to a mean of 340 s (Figure 4A). Preincubation of plasma with PdSP15a before addition of polyP gave a concentration-dependent increase in the clotting time, producing values of ≈550 s at a concentration of 5 μmol/L and 600 s at a concentration of 20 μmol/L of inhibitor protein (Figure 4A).

To determine whether PdSP15a could block the cleavage of HK after activation by DS, we supplemented human plasma with Abz-GFSPFRSVTQ-EDDnp, an intramolecularly quenched mouse HK–mimetic peptide.²⁸ This substrate was synthesized under the assumption that DS would sequentially activate FXII and prekallikrein, generating kallikrein, which would in turn hydrolyze the C-terminal ﬂanking sequence of bradykinin. As predicted, we found that the substrate was rapidly hydrolyzed in citrated human plasma supplemented with DS at a concentration of 10 μg/mL. As shown in Figure 4C, PdSP15a was not able to block the hydrolysis when tested at 2 and 5 μmol/L. However, at 10 μmol/L, PdSP15a prevented hydrolysis, reducing it to control levels (plasma plus substrate in the absence of DS; Figure 4C). We next repeated these assays without the preincubation step (ie, we mixed PdSP15a with DS shortly before addition to plasma). Again, we found that 10 μmol/L of the inhibitor abolished hydrolysis (Figure 4D). Additional controls showed that DS-induced hydrolysis was inhibited by the kallikrein inhibitor trans-4-aminoethylcloclohexanecarbonyl-l-phenylalanyl-l-aminophenyl acetic acid...
We did not detect significant hydrolysis in FXII-deficient plasma treated with DS (data not shown). Together, these results suggest that PdSP15a is able to attenuate contact phase activation by DS in plasma.

Induction of bradykinin production in skin has been demonstrated previously after activation of the contact pathway by intradermal injection of heparin and polyP. We measured the effect of PdSP15b on the degree of polyP-induced plasma extravasation by observing plasma leakage in mice first injected with Evans blue dye. The dye allows visualization and quantification of plasma leakage in the skin surrounding an injection site. Injections of 1.5 mmol/L polyP (P45) produced extravasation surrounding the injection site as measured by spectrophotometry after extraction. This was significantly inhibited (P<0.03; 2-tailed t test) by mixing an equal quantity of polyP with PdSP15b at a concentration of 20 μmol/L before injection (Figure 4B).

The results of the enzymatic assays described above suggested that PdSP15 proteins inhibit interaction of protein components of the pathway with anionic polymers. To measure this directly, we analyzed interactions of potential binding partners using gel filtration chromatography,
isostructural titration calorimetry, and surface plasmon resonance. PdSP15b binding with FXII was evaluated directly by gel filtration chromatography where changes in the retention volume of individual components are taken as indicators of binding. FXII was mixed with PdSP15b at a sodium chloride concentration of 0.15 mol/L and subjected to gel filtration chromatography under the same buffer conditions. No change in the retention volume of either FXII or PdSP15b was observed when compared with chromatograms of the individual components passed over the column separately (Figure 5A–5C). This indicates that PdSP15b does not form a high affinity complex with FXII that remains associated during chromatography. Accordingly, no significant interaction was observed between immobilized PdSP15b and FXII, FXIIa, FXI, Fkallikrein, or kallikrein in surface plasmon resonance experiments (Figure 5G).

Gel filtration chromatography was also used to detect binding of PdSP15b with DS and heparin. PdSP15b was mixed with DS or heparin in the same buffer as described above. In both cases the peak attributable to the inhibitor disappears completely from the chromatogram, indicating that all PdSP15b is tightly bound to both DS and heparin (Figure 5D and 5E). Heparin binding was further indicated by the fact that PdSP15b bound tightly to a heparin Sepharose column (Figure 5F). The protein could be eluted with NaCl (≈0.8 mol/L) indicating that binding is electrostatic in nature. When PdSP15b was immobilized on a surface plasmon resonance sensor chip, and DS was passed over this surface, saturable, concentration-dependent binding was observed with negligible dissociation being observed after injection (Figure 5H). This behavior is consistent with the multiple-binding site interaction expected when passing high-molecular-weight DS over the immobilized protein. This surface could be completely regenerated by injection of 400 nmol/L NaCl, again indicating that binding was electrostatic in nature. When biotinylated heparin was immobilized and PdSP15b was passed over the surface, binding was observed, and a dissociation constant \( K_d \) of 139 nmol/L was obtained from fitting of the concentration dependence of the steady-state resonance value (Figure 5I and 5J).

PolyP and heparin binding with PdSP15a were quantified using isothermal titration calorimetry in an experiment where solutions of either ligand were injected into the calorimeter cell containing PdSP15a. In both cases, the binding reaction was exothermic, and fitting to a single site binding model yielded association equilibrium constants \( K_a \) of 1.0±0.05×10^5 per mol/L for polyP (P45) and 8.9±1.6×10^6 per mol/L for heparin (considering an average molecular weight of 17000; Figure 5L). The binding stoichiometry of 12 binding sites per protein molecule for polyP suggests that a minimal binding site may include >10 phosphate units. The calculated

Figure 3. PdSP15s do not inhibit the amidolytic activity of factor Xlla (FXIIa), factor Xla (FXIa), or kallikrein but inhibit the polyP-mediated activation of FXI by thrombin and the dextran sulfate (DS)–mediated activation of FXI by FXIIa. A to C, Hydrolysis of chromogenic substrates by FXIIa (A), FXIa (B), and kallikrein (C) in the presence of increasing concentrations of PdSP15b. The initial reaction rate for each inhibitor concentration was normalized to a value of 1.0 in the absence of inhibitor. FXIIa activity was measured using S-2302, whereas FXIa and kallikrein activities were measured using S-2366. D, Inhibition of FXI activation by thrombin in the presence of polyP (P700). The activation of FXI (30 nmol/L) by α-thrombin (5 nmol/L) in the presence of 0.2 μmol/L polyP was evaluated after 20 minutes incubation at 37°C. Reactions were performed in the presence of PdSP15a concentrations ranging from 0 to 500 nmol/L. The FXIa product was quantified using the substrate S-2366. E, An experiment similar to that shown in D performed in the absence of polyP, but at an α-thrombin concentration of 15 nmol/L. F, Inhibition of the activation of FXI by FXIIa in the presence of DS. The activation of FXI (20 nmol/L) by FXIIa (0.8 nmol/L) in the presence of 0.2 μg/mL DS was evaluated after 30 minutes incubation at 37°C. At the end of each incubation period, FXIa was inhibited with corn trypsin inhibitor and the FXIa product was quantified using the substrate S-2366. This experiment was performed in the presence of increasing concentrations of PdSP15b between 0 and 2 μmol/L. G, The experiment in F was repeated in the absence of DS. FXIa was quantified after adding corn trypsin inhibitor and DS (0.2 μg/mL). For all assays the points represent the mean (±SE) of 3 replicates.
stoichiometry of 0.18 for heparin suggests that at least 5 to 6 protein molecules bind with each molecule of heparin (molecular weight of \( \approx 17000 \)).

The structure of PdSP15b was determined using x-ray diffraction methods to identify the probable structural determinants of anionic surface binding (Figure 6; Table). The structure consists of 6 \( \alpha \)-helices designated A–F arranged in a bundle that is stabilized by 3 disulfide bonds (Figure 6A and 6B). One disulfide bond links Cys 7 of \( \alpha \)-helix A with Cys 14 of \( \alpha \)-helix B, a second disulfide links Cys 20 of \( \alpha \)-helix B with Cys 88 of \( \alpha \)-helix E, and a third disulfide links Cys 71 of \( \alpha \)-helix D with Cys 97 of \( \alpha \)-helix E.

PdSP15a and b belong to the insect odorant-binding protein family, which consists mainly of ligand-binding proteins found in olfactory and gustatory sensory organs. The structure has some similarity to that of the biogenic amine/eicosanoid-binding D7 family from the salivary glands of mosquito species \(^{30,31}\) (Figure 6F and 6G). Superposition of PdSP15b with D7R4, a typical D7 protein from the mosquito \( \text{Anopheles gambiae} \), shows that the 2 have related topology but differ in...
Figure 5. Binding interactions of PdSP15a and b with factor XII (FXII), dextran sulfate (DS), polyphosphate, and heparin. A to C, Analysis of FXII-PdSP15b interactions using gel filtration chromatography. FXII alone (57 μg; A), a mixture of FXII (57 μg) and PdSP15b (64 μg; B), or PdSP15b alone (64 μg; C) in 20 mmol/L Tris HCl, pH 8.0, 150 mmol/L NaCl were applied to a Superdex-75 column and eluted with the same buffer. The chromatograms show no significant change in the elution characteristics of the 2 individual proteins when they were mixed, indicating a lack of interaction. D, Elution of DS (dashed line), PdSP15b (dash dot dot line), and a mixture of the 2 (solid line) from a Superdex-75 column using the elution buffer described above. The PdSP15b peak disappeared completely when mixed with DS, indicating binding between the 2. E, An experiment performed as in D, with heparin (dashed line) substituted for DS. The peak for PdSP15b alone (dash dot dot line) disappears when the protein is mixed with heparin (solid line). F, Chromatography of PdSP15b on a heparin Sepharose column eluted with a sodium chloride gradient as indicated in the figure. The protein was eluted with ≈850 mmol/L NaCl as shown by the right-hand axis. G, Surface plasmon resonance analysis of coagulation factor binding to PdSP15b immobilized on a CM5 sensor chip using amine coupling chemistry. FXII, FXIIa, factor XI (FXI), FXIa, prekallikrein, and kallikrein (traces displayed in the order shown on the panel) each at a concentration of 200 nmol/L were passed over the surface in 10 mmol/L HEPES pH 7.4, 0.15 mol/L NaCl, 0.5% surfactant P20. None produced a significant resonance response. H, DS was passed over a surface of PdSP15b prepared in a similar manner to the surface in G. Concentrations of DS ranging from 0 to 500 nmol/L were tested and are indicated on the plot. I, PdSP15b was passed over a biotinylated heparin surface prepared as described in the Materials and Methods in the online-only Data Supplement. Concentrations of PdSP15b (0–1000 nmol/L) are shown on the plot. J, Equilibrium steady-state resonance levels for each concentration shown in I are plotted against the concentration of PdSP15b, yielding a dissociation constant (K_d) of 139 nmol/L. K, Isothermal titration calorimetry (ITC) analysis of PdSP15a binding with polyP (P45). The concentration of protein in the calorimeter cell was 20 μmol/L in
the positions and number of helical elements (Figure 6F and 6G). The D7 proteins characteristically contain 8 α-helices designated A–H that surround a central pocket forming the ligand-binding site. Comparison of the D7R4 and PdSP15b structures shows that PdSP15b lacks α-helices B and H of D7R4 (Figure 6F and 6G). The region of helix B is shortened to a disordered turn structure containing 4 residues, and the C-terminal end of the protein is truncated resulting in the loss of α-helix H. The positions of the remaining elements are somewhat similar with a superposition of PdSP15b and a model of D7R4 lacking α-helices B and H producing a root-mean-square deviation of 3.8 Å for 67 Cα atoms. Because these 2 elements are missing in the PdSP15 proteins, no well-formed binding pocket is present, and it is unlikely that these proteins serve as binders of small molecule ligands (Figure 6F and 6G).

The electrostatic nature of anionic polymer binding by the PdSP15 proteins, as indicated by the salt sensitivity of interactions with heparin and dextran sulfate in chromatographic and surface plasmon resonance studies, suggests that a positively charged surface would be the most likely interaction region. In PdSP15b, the amphipathic α-helix D is lengthened relative to the D7s and studded with basic residues on its solvent-facing surface (Figure 6A–6E). Eight lysines and 3 arginines occur on the solvent-exposed side of this helix, whereas only 2 acidic residues, Asp 70 and Glu 78, are present (Figure 6C). Electrostatic calculations reveal a large, positively charged surface centered on α-helix D that lies at the edge of the overall protein structure, which is flattened and disk-like (Figure 6D and 6E). The highly basic nature of this region is conserved in the sequences of PdSP15a and b (Figure I in the online-only Data Supplement). Only Lys 81 in PdSP15a is replaced by threonine in PdSP15b, and Lys 74 in PdSP15b is replaced by glutamine in PdSP15a. Arrays of basic residues aligned along helical segments to give highly positively charged surface have been shown to be essential for

Figure 6. The crystal structure of PdSP15b. A, Ribbon diagram of the PdSP15b model with the α-helices labeled A–G based on correspondence with the α-helices of the mosquito protein D7R4. The side chains of basic residues (lysine and arginine) on α-helix E are shown as stick diagrams. B, The structure of A rotated 90° around the axis shown, putting the basic α-helix E in the foreground. C, α-Helix E shown in an expanded view oriented as shown in B with the basic residues shown as a stick diagram and labeled. D, Electrostatic surface model of PdSP15b oriented as in A. More positively charged parts of the surface are indicated as blue, more negatively charged parts of the surface are colored red, and neutral areas are colored white. Electrostatic calculations were performed using the program APBS. E, Electrostatic surface model oriented in the same way as the ribbon diagram in B. The model is colored as in D, F, Ribbon diagram of PdSP15b oriented to match D7R4 in G. The 6 α-helical elements are labeled A–G, highlighting the absence of α-helices B and H in D7R4 and the consequent absence of a small-molecule binding site. F, Ribbon diagram (green) of the mosquito serotonin-binding protein D7R4 with a bound serotonin molecule shown as a stick diagram (red). The α-helical elements are labeled A–H. The binding pocket for serotonin is formed largely from elements of α-helices B and H.
glycosaminoglycan interactions in other proteins, including antithrombin-III and apolipoprotein E.\textsuperscript{32,33}

**Discussion**

During feeding, salivary proteins of blood feeders limit host hemostatic and inflammatory responses to facilitate the intake of blood. Some of these proteins remove small-molecule mediators of mast cell and platelet activation from the area of a bite by binding them with high affinity. Mechanisms involving the sequestration of effectors and the coating of activating surfaces are commonly used by blood-feeding arthropods for inhibition of hemostasis and inflammation. Salivary proteins binding histamine, serotonin, norepinephrine, ADP, cysteinyl leukotrienes, leukotriene B\textsubscript{4}, thromboxane A\textsubscript{2}, and phosphatidylserine have been identified and shown to inhibit platelet activation, coagulation, and vasoconstriction in vitro and in vivo.\textsuperscript{34,35} Aegeyptin, a mosquito protein that coats collagen surfaces, blocks the binding of von Willebrand factor and the interaction of collagen with glycoprotein VI, the major platelet collagen receptor.\textsuperscript{36} Many of these binding proteins have been crystallized in the presence of ligands, revealing sophisticated and highly selective ligand-binding sites.\textsuperscript{30,34,37,38} Here, we have extended the functional range of salivary scavengers to include the sequestration of binding sites for coagulation factors on heparin and polyP, 2 proven endogenous activators of coagulation and inflammation. We have demonstrated that PdSP15a and b bind these polymers and effectively inhibit the contact activation of FXII, the contact activation of FXI, the activation of FXI by thrombin and FXIIs, and the reciprocal activation of FXII and prekallikrein initiated by DS.

The amount of protein present in the salivary glands before feeding is small (ca. 1 μg), but the volume of blood ingested by a blood feeder is also very small (ca. 1 μL for a female sand fly), as is the area of skin damaged by the bite.\textsuperscript{39,40} Ligand-scavenging proteins pumped into the feeding space are thought to reach concentrations comparable with those of their target agonists because between 25% and 85% of salivary gland contents are expelled during a single feeding.\textsuperscript{40,41} Because scavenger proteins act by stoichiometrically binding relatively abundant agonist molecules, they tend to be among the most abundant in the saliva.\textsuperscript{40} Consistent with this, measures of transcript abundance and protein concentration have shown that PdSP15s are the most concentrated proteins in the salivary mixture by a factor of >2.\textsuperscript{24} We have shown that inhibition of polyP-mediated FXII activation in a manner consistent with an anionic polymer–binding mechanism can also be attained using salivary gland extracts at a protein concentration of 5 to 20 ng/μL, a level well below the protein concentration reached in the volume of the blood meal.

The PdSP15s seem to function in vivo as inhibitors of bradykinin formation because sand fly saliva contains a potent inhibitor of FXa (lufaxin and its relatives) that seems to act as the major anticoagulant.\textsuperscript{39} However, salivary mixtures are known to inhibit physiological processes (such as platelet activation) by attacking >1 activating pathway simultaneously.\textsuperscript{40} The PdSP15s may, therefore, also act redundantly in the inhibition of coagulation or thrombosis, particularly because the importance in normal hemostasis of processes such as the thrombin-mediated activation of FXI is still being established. Along with other anti-inflammatory proteins in the salivary mixture, PdSP15s most likely act primarily to delay the swelling and sensation of pain associated with the bite, thus avoiding defensive responses from the host.\textsuperscript{40} We also cannot rule out the possibility that PdSP15s play additional roles, and that anionic surface binding serves as a means of targeting the protein to specific locations, such as the glycosaminoglycan-covered surfaces of endothelial cells or the anionic phospholipid–containing surface of activated platelets.\textsuperscript{41}

The importance of contact pathway inhibition in blood feeding is reflected by the fact that several recently described salivary inhibitors of FXIIa have shown efficacy as antithrombotics and anti-inflammatories in model systems. For example, infestin and Ir-CPI, from the insect Triatoma infestans and the tick Ixodes ricinus, respectively, bind FXIIa directly and inhibit thrombosis in vivo. Infestin is a Kazal-type serine
protease inhibitor that shows specificity for FXIIa, whereas Ir-CPI is a Kunitz-type inhibitor that also inhibits the activity of FXIa and kallikrein. In both cases, occlusive thrombus formation was prevented by these proteins in animal models without an inhibitory effect on hemostasis. The efficacy of an anionic surface–binding mechanism of inhibition has been established more recently in studies where small molecule and macromolecular inhibitors of thrombosis are shown to specifically target anionic surfaces and prevent activation of the contact pathway. Various polycationic compounds, such as polybrene, polyamine dendrimers (eg, PAMAM G1-G5), polyethylenimine, spermine, and protamine, bind polyP and nucleic acids with submicromolar affinity and effectively inhibit thrombosis and vascular leakage in vivo in rodent models. The same types of compounds have also been shown to inhibit inflammation by limiting the nucleic acid–mediated activation of toll-like receptors. The PdSP15 proteins are naturally occurring substances that inhibit hemostatic and inflammatory processes by a mechanism similar to these. The inhibition of coagulation initiated with the colloidal silica activated partial thromboplastin time reagent suggests that they are relatively nonspecific and can block binding sites on a variety of potential activating polymers. The compact, all-helical PdSP15 proteins contain an amphipathic helical segment that contributes to a positively charged surface that is the most likely site of electrostatic interaction with all of these anionic materials.

The targeting of surfaces rather than specific proteins provides the PdSP15 proteins with the potential to inhibit multiple processes in hemostasis and inflammation. In addition to the activation of FXII, anionic surfaces serve to stabilize complexes of FXIIa with FXI, HK and prekallikrein, as well as the complex of thrombin with FXI. They are also known to inhibit the activation of tissue factor pathway inhibitor by stimulating the activation of FV, stabilize fibrin clots, and modulate the interaction between von Willebrand factor and the platelet receptor glycoprotein Ib. PdSP15 proteins inhibit the activation of FXII and FXI, as well as the cleavage of FXI by FXIIa or thrombin, and would be expected to inhibit any anionic surface–mediated reaction. Mast cell proteases such as tryptase occur as heparin complexes, with heparin being essential for stabilization of the tetrameric structure of the enzyme. Perhaps these complexes could also be disrupted by heparin-binding proteins. The recognition of a variety of anionic substances, including glycosaminoglycans, and polyp, by the PdSP15 proteins suggests that they could be effective as both anticoagulants and anti-inflammatory molecules in both the circulation and the skin. The inhibition of coagulation, the cleavage of kininogen mimetic peptides in plasma, and plasma extravasation in skin show that the PdSP15 proteins are capable of inhibiting surface-mediated reactions in complex biological mixtures in vivo and may have properties that are useful therapeutically.

*P. duboscqi* transmits *Leishmania* parasites during feeding, and the inflammatory effects of salivary components have been shown to influence establishment of the parasite in the skin. Host delayed-type hypersensitivity responses to salivary proteins create a hostile environment for *Leishmania*, and some salivary proteins can serve as protective antigens. The anti-inflammatory effects of native salivary components could also affect parasite establishment, and with some pathogens arthropod salivas have been shown to enhance infectivity. The reasons for this enhancement are unclear in most cases and may be related to the functional properties of salivary proteins. Maxidilan, a vasodilator peptide from another sand fly species, exacerbates *Leishmania* infections and has been found to bind to the PAC1 receptor for the pituitary adenylyl cyclase–activating polypeptide receptor and influence cyto-kine levels at the bite site. Modulation of the environment of the skin and circulation by the combined effects of many salivary proteins may play an important role in the establishment of vector-borne pathogens.

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**Disclosures**

None.

**References**

11. Muller F, Mutch NJ, Scheck WA, Smith SA, Esterl L, Sprong HM, Schmidtbaue S, Gahl WA, Morrissey JH, Renné T. Platelet...
42. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. Proc Natl Acad Sci USA. 2010;107:903–908.
44. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. Proc Natl Acad Sci USA. 2010;107:903–908.
47. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. Proc Natl Acad Sci USA. 2010;107:903–908.


### Significance

Blood-feeding insects transmit a variety of parasitic, viral, and bacterial disease agents when they ingest blood from a mammalian host. Proteins in the saliva of these insects facilitate feeding by inhibiting physiological processes acting to prevent blood loss and inflammation. In this study, we show that 2 salivary proteins from the sand fly, *Phlebotomous duboscqi*, inhibit autoactivation of coagulation factor XII, a reaction that occurs when the enzyme precursor binds to anionic polymers. This is the initial step in the contact pathway of coagulation leading to activation of the coagulation cascade and formation of the proinflammatory peptide bradykinin. This pair of proteins has been given the name PdSP15 and shown to act by binding tightly with the polymers heparin and polyphosphate thereby preventing interaction with the coagulation factor. The likely physiological function of this mechanism would be the inhibition of the proinflammatory activities of bradykinin, particularly pain and vascular leakage.
Novel Family of Insect Salivary Inhibitors Blocks Contact Pathway Activation by Binding to Polyphosphate, Heparin, and Dextran Sulfate

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Supplemental Materials and Methods

Materials

DS (MW=500,000), porcine mucosal heparin, sodium phosphate glass (polyP P45, a mixture of polyphosphate chain lengths with an average size of 45 monomer units), biotinylated heparin, polybrene and Evans Blue dye were obtained from Sigma-Aldrich. PolyP (P700, a mixture of polyphosphate chain lengths with a modal size of 700 monomers) was obtained from Kerafast. Neutravidin was obtained from Thermo-Fisher and reagents for surface plasmon resonance were obtained from GE Healthcare. Human PK, kallikrein, FXII, FXI, FXIIa and FXIa were obtained from Enzyme Research Laboratories. Corn trypsin inhibitor and human α-thrombin were obtained from Chromogenex, were obtained from Diapharma. Chromogenic substrates manufactured by Chromogenics, were obtained from American Diagnostica and Diagnostica Stago. Coagulation factor-deficient plasmas were obtained from American Diagnostica.

Protein expression and purification

The cDNA for PdSP15a was obtained from a previously described salivary gland library of *Phlebotomus duboscqi* 1. The position of the signal sequence cleavage site was predicted using the program Signal-P and agreed with published data from Edman degradation of salivary gland-derived proteins 1. The PdSP15b cDNA was prepared synthetically. The signal sequences were removed from both cDNAs using PCR and were replaced by an initiator methionine codon. cDNAs for PdSP15a (GenBank accession ABI15933) and PdSP15b (GenBank accession ABI15943) lacking signal sequences were cloned into the expression vector pET17b and moved into the *Escherichia coli* cell line BL21(DE3)pLysS for expression. Inclusion bodies were produced for each protein using methods described previously 2. The inclusion bodies were dissolved in 6 M guanidine HCl, pH 8.0 and refolded by dilution into a large excess 20 mM Tris HCl, pH 8.5, 300 mM arginine HCl. After concentration, the proteins were purified by gel filtration chromatography on Sephacryl S-100 and cation exchange chromatography on SP-Sepharose. To prepare salivary gland extracts, female *P. duboscqi* glands were dissected five days after emergence and disrupted by sonication in 1 µL phosphate-buffered saline per salivary gland pair. After centrifugation at 15,000 x g the extract contained 1.06 µg/µL protein as measured by the BCA assay.

Inhibition of coagulation in human plasma

The inhibitory effect of PdSP15s on the activated partial thromboplastin time (aPTT) was measured using 30 µL of human plasma pre-incubated (5 min at 37°C) with PdSP15a or b (0-10 µM), followed by the addition of 30 µL of Cephen (Aniara) aPTT reagent diluted ten-fold from the standard concentration in 20 mM Tris pH 7.4, 150 mM NaCl (reaction buffer A). Cephen provides an inorganic activating surface that is equivalent to colloidal silica. After an incubation period of 3 min at 37°C coagulation was initiated by adding 30 µL 25 mM CaCl₂, and the onset of clotting was detected at 37°C by measuring changes in turbidity (as indicated as the point of half-maximal increase in absorbance at 650 nm) in a Thermomax plate reader (Molecular Devices).

The effect of PdSP15a on clotting time triggered by polyP (P700) was tested as described by Smith et al. 3 with some modification. Briefly, different concentrations of PdSP15a in reaction buffer A were incubated with polyP at a final concentration of 5 µg/mL, for 5 minutes
at 37°C, in a reaction volume of 50 µL. Subsequently, 50 µL of normal reference human plasma was added, and after three additional minutes of incubation, 50 µL of 25 mM CaCl₂ was added. Clotting times at 37°C were then measured using a Start4 coagulometer (Diagnostica Stago).

**Reciprocal activation of FXII and PK**

Inhibition of reciprocal activation of FXII and PK was measured by pre-incubating PdSP15a and b (0-10 µM) with 0.2 nM FXII for 15 min at 37°C in reaction buffer A containing 0.3 % bovine serum albumin (BSA), followed by addition of PK (10 nM) and DS (0.2 µg/mL) ⁴. After 10 minutes of reaction at 37°C, the reaction volume was increased to 100 µL by adding buffer A plus BSA and chromogenic substrate S-2302 (250 µM final concentration) to each well. Hydrolysis of the substrate was observed at 405 nm at 37°C in a Versamax plate reader (Molecular Devices). Initial reaction rates were expressed as percentage of the control activity (in the absence of PdSP15a and b).

**Coagulation factor assays**

Contact activation of FXII was detected by hydrolysis of the chromogenic substrate S-2302 in the presence of PdSP15a and b. In a total volume of 100 µL reaction buffer A, PdSP15a or b (0-8 µM) were added, followed by either DS (1.0 µg/mL), heparin (10 µg/mL) or polyP (P700, 52.5 µM as monomeric phosphate). After an incubation period of 10 min, FXII was added to a concentration of 140 nM, followed by the chromogenic substrate S-2302 (0.4 mM final concentration). The production of FXIIa was observed continuously over a period of 1-2 hr as an increase in absorbance at 405 nm at a temperature of 37°C in a Thermomax plate reader. The contact activation of FXI in the presence of 1.0 µg/mL DS was measured in the same manner as FXII, but with a final FXI concentration of 200 nM. In this case FXIa production was monitored continuously using the substrate S-2366 at a concentration of 0.25 mM.

Inhibition of the amidolytic activity of individual coagulation factors was evaluated in reaction buffer A containing 0.3 % BSA. FXIa (1 nM), FXIIa (10 nM) or kallikrein (10 nM) were incubated with different concentrations (0-8 µM) of PdSP15b for 5 minutes at 37°C. After incubation, the respective chromogenic substrate (S-2366, for FXIa and kallikrein or S-2302, for FXIIa) at a final concentration of 0.25 mM was added and reaction progress was measured at 37°C at 405 nm in a Versamax plate reader.

Inhibition of thrombin-mediated activation of FXI in the presence and absence of polyP was performed as described by Choi et al. ⁵. Concentrations of PdSP15a ranging from 0-500 nM, were pre incubated with 2 µM polyP (P700) for 10 minutes at 37°C in a buffer consisting of, 30 mM HEPES pH 7.3, 50 mM NaCl and 0.1 % BSA. This was followed by addition of 5 nM α-thrombin and 30 nM human FXI, and samples were then incubated for 0 or 20 minutes at 37°C, at which point the reactions were stopped by addition of polybrene (6 µg/mL), to scavenge polyP, and 10 nM anophelin, to inhibit thrombin ⁶. In order to quantify the generated FXIa after incubation, S-2366 was added to a concentration of 0.25 mM and reaction progress was measured at 37°C at 405 nm in a Versamax plate reader. The quantities of FXIa produced were
determined using a standard curve generated with known concentrations of FXIa. To determine the degree of inhibition in the absence of polyP, the reactions were performed as described above, but without the addition of polyP, and with an α-thrombin concentration of 15 nM and anophelin concentration of 20 nM.

In a similar manner, inhibition of FXIIa-mediated activation of FXI was measured in the presence and absence of DS. PdSP15b (0-8 µM) was added to 100 µL reaction buffer A, followed by addition of DS (0.2 µg/mL), FXIIa (0.8 nM), and FXI (20 nM). The plate was incubated at 37°C for 30 min and the reaction was stopped by adding corn trypsin inhibitor (500 ng/mL), which selectively inhibits FXIIa. The FXIa concentration was determined by measuring the hydrolysis of S-2366 (0.5 mM) at 37°C in a plate reader. The reaction of FXIIa with FXI was also performed in the absence of DS. After the incubation period and the addition of corn trypsin inhibitor, DS was added to a concentration of 0.2 µg/mL prior to addition of chromogenic substrate. The production of FXIa was quantified using a standard curve generated using commercially prepared FXIa.

**Inhibition of cleavage of a kininogen mimetic peptide in plasma**

The activation of FXII/PK in plasma was evaluated using hydrolysis of a kininogen mimetic peptide by kallikrein as a read-out. Internally quenched fluorescent substrates in the form of mouse kininogen fragments containing C-terminal flanking region for BK were synthetized as described previously. The hydrolysis of the substrate Abz-peptidyl-EDDnp (Abz = o-aminobenzoyl and EDDnp = ethylenediamine 2,4-dinitrophenyl) was monitored by measuring the fluorescence at λ<sub>ex</sub> = 320 nm and λ<sub>em</sub> = 420 nm in a Hitachi F-4500 fluorescence spectrophotometer. Hydrolysis conditions were: 20 mM Tris/HCl and 1mM EDTA, pH 7.4, citrated human plasma 1:20, 4 µM substrate, 10 µg/mL DS 500 kDa. The assay was carried out in the presence or absence of PdSP15a at concentrations of 2, 5 and 10 µM.

**Inhibition of BK-mediated plasma extravasation**

Animals were housed under pathogen-free conditions at the National Institute of Allergy and Infectious Diseases (NIAID) Twinbrook animal facility in Rockville, Maryland. All animal experimental procedures were reviewed and approved by the NIAID Animal Care and Use Committee. Swiss mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (80 mg/kg and 10 mg/kg) and intravenously injected in the tail vein with 100 µL of sterile Evans blue solution in phosphate-buffered saline (PBS). In the subscapular area of the back, mice were given intradermal injections of PBS, PdSP15b (20 µM) in PBS, polyP (P45, 1.5 mM) in PBS, or polyP (P45, 1.5 mM) in PBS mixed with PdSP15b (20 µM). After 30 minutes, the mice were euthanized and the skin was removed and 0.5 cm diameter disks were removed at the injection sites for extraction of Evans blue in manner similar to that described by Muller et al.. The dye was extracted by soaking the disks in 1 mL N,N-dimethylformamide overnight at a temperature of 37°C. Extravasation was quantified by measuring the absorbance of the dimethylformamide solutions at 639 nm.

**Chromatographic binding assays**

For chromatographic detection of PdSP15b –FXII complexes, 57 µg human FXII was incubated for 10 minutes with 64 µg PdSP15b in a volume of 200 µL 20 mM Tris HCl, pH 8.0, 150 mM NaCl. The mixture was then injected onto a Superdex-75 column (10/30) and eluted with the same buffer. For comparison, the same concentrations of FXII and PdSP15b alone were separately passed over the column under the same conditions.
In order to detect interaction of the inhibitor with DS, PdSP15b (200 µg) was mixed with 25 µg DS in 300 µL of 20 mM Tris HCl, pH 8.0, 150 mM NaCl, and after an incubation of period of 5 min the mixture was injected onto a Superdex-75 column (10/30) and eluted with the same buffer. An identical procedure was also used to measure heparin binding with 200 µg of PdSP15b and 200 µg of heparin.

**Isothermal titration calorimetry and surface plasmon resonance**

Isothermal titration calorimetry (ITC) was carried out with a VP-ITC microcalorimeter (Microcal) at 30 °C using 10 µL of injections polyP or heparin solution into a solution of PdSP15a contained in the calorimeter cell. The protein was diluted to 20 µM in 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, while polyP (P45) and heparin were dissolved in the same buffer at concentrations of 4.5 mM and 200 µM, respectively. All samples were degassed prior to use. After conversion of the injection heats to enthalpies with heats of dilution subtracted, the data were fitted to a single-site binding model using the Microcal-Origin software package.

Surface plasmon resonance (SPR) was performed using a Biacore T-100 instrument. PdSP15b at a concentration of 15 µg/mL in 10 mM sodium acetate, pH 5.0 was passed over a CM-5 sensor chip (GE Healthcare) surface and immobilized using amine coupling chemistry. Coagulation factors and DS were passed over these surfaces in a buffer consisting of 10 mM HEPES pH 7.4, 0.15 M NaCl, 0.5 % surfactant P20. For experiments with DS, the sensor surface was regenerated with a short pulse 0.5 M NaCl, and for experiments with coagulation factors, the surface was regenerated with a short pulse of 10 mM glycine pH 2.5. Biotinylated heparin was bound after immobilizing neutravidin (40 µg/mL) on a CM5 sensor surface in 10 mM sodium acetate, pH 5.0 by passing the solution over the surface for 7 min at a flow rate of 10 µL/min. Heparin was bound to the desired level using manual injections of the ligand in 10 mM HEPES pH 7.4, 0.15 M NaCl, 0.5 % surfactant P20. PdSP15b was passed over this surface in 10 mM HEPES pH 7.4, 0.15 M NaCl, 0.5 % surfactant P20 and the dissociation constant was obtained by measuring the resonance response at the steady-state and fitting these values to a hyperbolic function using the Biacore evaluation software.

**Crystallization and diffraction data collection**

Recombinant PdSP15b was crystallized using the hanging drop vapor diffusion method in a solution consisting of 1.6 M ammonium sulfate, 0.1 M MES, pH 6.0. For the purpose of producing a selenomethionine derivative of PdSP15b, the expression construct described above was moved in to the methionine auxotrophic *E. coli* strain B834(DE3)pLysS and grown in SelenoMet medium (Molecular Dimensions) supplemented with selenomethionine. The protein was produced as inclusion bodies, refolded, and purified as described above. The purified selenomethionine derivative crystallized under the same conditions as the wild type protein. For data collection, the crystals were flash frozen in liquid nitrogen after transfer to a cryoprotectant containing 3.7 M ammonium sulfate, 0.05 M MES, pH 6.0.

Data collection was performed at beamlines 22-BM and 22-ID at the Southeast Regional Collaborative Access Team (SER-CAT) Advanced Photon Source (APS), Argonne National Laboratory. Native PdSP15b diffracted to 2.6 Å resolution in the space group P2_12_1 with seven monomers in the asymmetric unit. The selenomethionine derivative of PdSP15b crystallized in the same form and diffracted to a resolution of 3.3 Å.
The structure of PdSP15b was determined using single anomalous diffraction (SAD) methods with diffraction data from crystals of the selenomethionine derivative of the protein. Data collected at the peak absorption wavelength for selenium were integrated and scaled using HKL2000. Identification of selenium sites and phasing were performed using SHELX C, D and E, as implemented in HKL2MAP. Backbone traces containing parts of most of the seven molecules in the asymmetric unit were built in an automated fashion using BUCCANEER, and the remaining portions were built manually using Coot. A pentameric model was first obtained using the selenomethionine data. The additional two monomers were located in the native asymmetric unit by molecular replacement with a monomeric model using PHASER. Refinement of the structure was carried out using phenix.refine. The coordinates for the wild type native structure of PdSP15b have been deposited with the RCSB protein data bank with the accession number 4JD9.


Supplemental Figure I. Amino acid sequence alignment of PdSP15a and b along with PpSP15 from *P. papatasi*. The alpha helical elements derived from the PdSP15b crystal structure are marked as bars above the alignment and labeled αA-G corresponding to Figure 6. The basic residues found in αE are highlighted in black.