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Characterization of anti-hemostatic factors in the argasid, *Argas monolakensis*: Implications for the evolution of blood-feeding in the soft tick family

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Abstract

To date, the only anti-hemostatic factors characterized for soft ticks are for *Ornithodoros moubata* and *O. savignyi*, ticks that feed mainly on mammals. This includes thrombin (ornithodorin and savignin), fXa (TAP and fXaI) and platelet aggregation (disagegin and savignygrin) inhibitors that belong to the BPTI-Kunitz protein family. This raises the question on how well anti-hemostatic factors will be conserved in other soft tick genera that feed on other vertebrates such as birds. We characterized the anti-hemostatic factors from *Argas monolakensis*, a soft tick that feeds mainly on Californian gulls. The main anti-clotting factor (monobin) is an ortholog of ornithodorin and savignin and shows similar slow tight-binding kinetics. The main anti-platelet activities are apyrase and fibrinogen receptor antagonists (monogrins). The monogrins are orthologs of disagregin and savignygrin and like savignygrin presents the RGD integrin-recognition motif on the BPTI substrate-binding presenting loop. This implies that the anti-hemostatic factors evolved in the ancestral soft tick lineage and has been maintained in soft tick species from two distinct genera with different host preferences. The *Argas* derived anti-hemostatic factors bind to mammalian targets with affinities similar to that observed for their orthologs in the *Ornithodoros* genus. This cross-reactivity could have facilitated the switching of soft ticks from avian to mammalian hosts and can explain in part the ability of *Argas* ticks, to feed on humans, thereby remaining a possible health risk.

Keywords

Argas monolakensis; blood-feeding; blood-clotting; evolution; platelet aggregation; ticks

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1. Introduction

Hematophagous arthropods 4.1 adapted several times independently to a 5.5 blood-feeding lifestyle (Ribeiro, 1995). In insects this occurred in the Orders Diptera (flies), Hemiptera (bugs), Phthiraptera (lice) and Siphonaptera (fleas) (Ribeiro, 1995). In arachnids, hematophagous behavior evolved independently in the main tick families and several times in Mesostigmatid mites (Mans and Neitz, 2004a; Radovsky, 1969). In all cases this adaptation involved the evolution of mechanisms to counter-act the vertebrate host's defenses. These mechanisms mainly include the secretion of salivary gland derived bio-active components into the feeding site (Ribeiro, 1995). Comparison of sialomes (salivary gland proteomes of hematophagous organisms) from different blood-feeding species corroborates their independent adaptation to a blood-feeding lifestyle (Ribeiro and Francischetti, 2003). In almost all cases the mechanisms to counteract the host's immune and hemostatic defenses differ between the major arthropod families, i.e. no orthologous relationships. In the case of the two major tick families, the Argasidae (soft ticks) and Ixodidae (hard ticks) this observation also holds (Mans and Neitz, 2004a). Thus far the protein families to which anti-hemostatic factors belong differ in hard and soft ticks (Mans et al. 2002a; Mans and Neitz, 2004a). Even within the tick families no orthologs for anti-hemostatic factors have yet been found between different genera, raising the question on how conserved anti-hemostatic mechanisms are within ticks. Finding orthologs shared between genera will allow us to trace anti-hemostatics back to their last common ancestor, thereby establishing points of reference regarding the evolution of blood-feeding in ticks. The present study does this by showing that soft tick anti-hemostatic mechanisms can be traced back to the ancestral argasid lineage.

Argasids are composed of three main genera, *Argas* (57 species), *Ornithodoros* (37 species) and *Carios* (Klompen et al. 1996; Barker and Murrell, 2004). Limited host specificity is observed for soft ticks and most will feed on a range of vertebrates, although certain broad preferences can be established for the different genera (Hoogstraal, 1985). As such, *Argas* sp. feeds mainly on birds, *Ornithodoros* sp. feeds mostly on mammals and *Carios* sp. feeds on birds, bats and mammals (Klompen et al. 1996; Barker and Murrell, 2004). Even so, while *Argas* ticks prefer avian hosts, it is known that they will feed on mammals when birds are absent and recent reports showed that they can cause severe allergic responses in humans (Sirianni et al. 2000; Rolla et al. 2004; Hilger et al. 2005; Quercia et al. 2005; Kleine-Tebbe et al. 2006; Spiewak et al. 2006). *Argas* ticks can thus modulate the mammalian hemostatic system. In fact, the first study that showed the presence of an anti-clotting factor in tick salivary glands showed that salivary gland extracts from *Argas persicus* could prolong clotting time of human whole blood (Nuttall and Strickland, 1909). This raises the question whether the anti-hemostatic mechanisms from *Argas* ticks might be evolutionary related to those found in genera that feeds preferentially on mammals. However, the nature of the anti-hemostatic factors found in *Argas* ticks has not been elucidated yet. Furthermore, knowledge of anti-hemostatics in *Argas* species will provide us with a better understanding of the evolution of blood-feeding in the soft tick family, as the current knowledge on anti-hemostatics from soft ticks is limited to a very small part of their species diversity. So far the only anti-hemostatics described in soft ticks are for *O. moubata* and *O. savignyi* (Gaspar

et al. 1995; Gaspar et al. 1996; Joubert et al. 1998; Mans et al. 2002b; Nienaber et al. 1999; van de Locht et al. 1996; Waxman et al. 1990; Karczewski et al. 1994; Mans et al. 2002c). These ticks are part of the “*Ornithodoros moubata*” complex and include a small set of ticks from the *Ornithodoros* genus that is mainly found in the Old World (Walton, 1964). To gain more insight into the evolution of anti-hemostatic mechanisms in the soft tick family, we investigated the anti-hemostatic mechanisms from *A. monolakensis*.

A. monolakensis lies phylogenetically deep within the *Argas* genus and is well separated from the Ornithodorinae, which includes the *Ornithodoros*, *Carios* and *Otobius* genera (Klompen, 1992; Black and Piesman, 1994). It is closely related to *A. cooleyi*, a parasite of cliff swallows (*Hirundo pyrrhonota*) but is limited to the islands located on Mono Lake (California) where it feeds annually in summer on breeding Californian gulls (*Larus californicus*) (Schwan et al. 1992). It will also feed readily on humans and has been a problematic feature for ornithologists working on the islands (Schwan and Winkler, 1984). It is also a suspected vector of Mono Lake virus (Rheoviridae: *Orbivirus*) (Schwan et al. 1988). We show that the main clotting inhibitor (monobin) found in *Argas* ticks is orthologous to the thrombin inhibitors from the genus *Ornithodoros*. Similarly the main platelet aggregation inhibitors (monograns), is orthologous to fibrinogen receptor antagonists from the genus *Ornithodoros*. This implies that these anti-hemostatic mechanisms were present in the ancestral argasid lineage. It also explains in part why *Argas* ticks can readily feed on humans, thereby remaining a health risk.

2. Materials and Methods

2.1. Tick collection and dissection of salivary glands and preparation of salivary gland extract (SGE)

A. monolakensis ticks were collected on Little Tahiti Island at Mono Lake, California on 8th September 2006. Salivary glands were dissected from female ticks in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4 by tearing the dorsal cuticle and removing the glands with forceps. Glands were washed in dissection buffer before being frozen at -70°C . Glands were reconstituted in 20 mM Tris-HCl, pH 7.4 and disrupted by sonication, before the cell debris were pelleted by centrifugation at 16000xg for 20 minutes on a bench top centrifuge.

2.2. Assay for clotting factors

Activated partial thromboplastin time (APTT) was performed as described using the activated partial thromboplastin time reagent (Helena Laboratories, Beaumont, TX) (Calvo et al. 2006). Briefly, 50 μl of normal plasma was incubated with SGE (10 μl) for 5 min at 37°C , before addition of 50 μl APTT reagent (diluted 20X to give a clotting time of $\sim 100\text{s}$) pre-heated at 37°C . After incubation for 5 min at 37°C , pre-heated CaCl_2 (20 mM) was added and clotting was measured at 650 nm every 11s with shaking before and after each reading. Clotting time was measured at 50% of the total absorbance. The pro-thrombin time test (PTT) was performed using the thromboplastin reagent (Helena Laboratories, Beaumont, TX). Normal plasma (50 μl) and SGE (50 μl) was incubated at 37°C for 5 min, before addition of 50 μl pre-warmed PTT reagent (diluted 1000X with 20 mM CaCl_2 to give a clotting time of $\sim 80\text{s}$) after which clotting time was measured as described above. For

recalcification time of chicken plasma, 100 µl of plasma (Sigma-Aldridge, Inc) was incubated with 1 µl of various concentrations of SGE or recombinant monobin for 20 minutes before addition of 100 µl CaCl₂ (20 mM). Reagents were added to siliconized glasstubes and clotting was monitored every 5 minutes by inversion of the tube. Clotting time was noted when no plasma flow could be observed and plasma was immobilized in the glass tubes. Recalcification assays were performed with chicken plasma, as chickens lack a functional intrinsic pathway (APTT test) and the extrinsic pathway (PTT test) does not work with heterologous tissue factor (Frost et al. 1999). Under the conditions used chicken plasma only clotted after 15–20 minutes of incubation at 37°C compared to the APTT and PTT tests with human plasma that clotted within 100 seconds.

2.3. Chromogenic assays for serine proteases

Proteolytic activities were measured spectrophotometrically using protease specific chromogenic substrates as described previously (Nienaber et al. 1999). Inhibition of thrombin activity by SGE or during monitoring of purification was measured by incubating 10 µl sample and 90 µl alpha-thrombin (9 mU total, Enzyme Research Laboratories, IN) in incubation buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.1% BSA) at 37°C for 15 min. Chromogenic substrate (100 µl S2238 – diluted in incubation buffer to give a final concentration of 250 µM, Chromogenix) was added and the reaction measured at 405 nm for 10 minutes with constant mixing every 12 seconds at 37°C. Inhibition of fXa activity was measured by incubating 10 µl SGE and 90 µl fXa (0.7nM final concentration diluted in incubation buffer) for 10 minutes at 37°C before addition of S2222 (250 µM final concentration diluted in incubation buffer, Chromogenix) and monitoring the reaction as described above. In both cases the slope of the reaction was used to calculate the enzyme activity. Specificity for other serine proteases were determined as described previously (Nienaber et al. 1999; Francischetti et al. 2002).

2.4. Purification of monobin and the monogrins

Salivary gland extract (100 gland pairs/500 µl, ~1 mg total protein) prepared in buffer A (20 mM Tris-HCl, pH 7.4) was applied to a mono-Q HR 10/10 anion exchange column (Amersham Biosciences). Unbound protein were eluted over a five minute period, before bound protein were eluted using a gradient of buffer B (20 mM Tris-HCl, pH7.4, 1M NaCl) over 60 minutes. Protein elution was monitored at 280 nm and 1 ml fractions were collected. Fractions were tested for the inhibition of the APTT, PTT tests and the amyolytic activity of thrombin using the chromogenic substrate S2238. Fractions that showed anti-clotting activity were pooled and diluted in RP buffer A (0.1% TFA, 0.1% acetonitrile) and directly applied to a C4 reversed phase column (Vydac). Protein were eluted using a gradient of RP buffer B (0.1% TFA, 60% acetonitrile) over 60 minutes. Fractions were dried on a vacuum concentrator before resuspension in resuspension buffer (20 mM Tris-HCl, 0.15M NaCl, pH 7.4) before assaying for activity.

Fractions collected during the anion exchange chromatography run described above were tested for inhibition of platelet aggregation induced by ADP, collagen and epinephrine using the microplate method. Fractions were also tested for apyrase activity as described. The flow-through fractions obtained from the anion exchange column were directly applied in

buffer A onto a mono S cation exchange column (Amersham Biosciences). Unbound protein were eluted over a five minute period, before bound protein were eluted using a gradient of buffer B (20 mM Tris-HCl, pH7.4, 1 M NaCl) over 60 minutes. Protein elution was monitored at 280 nm and 1 ml fractions were collected and tested for inhibition of platelet aggregation induced by ADP, collagen and epinephrine using the microplate method. Fractions that showed anti-platelet activity were pooled and diluted in RP buffer A (0.1% TFA, 0.1% acetonitrile) and directly applied to a C4 reversed phase column (Vydac). Protein were eluted using a gradient of RP buffer B (0.1% TFA, 60% acetonitrile) over 60 minutes. Fractions were dried on a vacuum concentrator before resuspension in resuspension buffer (20 mM Tris-HCl, 0.15 M NaCl, pH 7.4) before assaying for activity.

2.5. Peptide mass fingerprinting

Purified monobin and monogrins (~30 pmoles) were alkylated and trypsin digested using standard mass spectrometry methods. Tryptic digests were analyzed by coupling the Nanomate (Advion BioSciences)—an automated chip-based nano-electrospray interface source—to a quadrupole time-of-flight mass spectrometer, QStarXL MS/MS System (Applied Biosystems/Sciex). Computer-controlled, data-dependent automated switching to MS/MS provided peptide sequence information. AnalystQS software (Applied Biosystems/Sciex) was used for data acquisition. Data processing and databank searching were performed with Mascot software (Matrix Science). The NR protein database from the NCBI, National Library of Medicine, NIH, was used for the search analysis, as was a protein database generated during the course of this work.

2.6. Recombinant expression of monobin

A salivary gland cDNA library of *A. monolakensis* was constructed as previously described (Valenzuela et al. 2002). After identification of the putative monobin gene by peptide mass fingerprinting and library scanning, a corresponding cDNA sequence were amplified using primers that allowed cloning into the Pet17b expression vector (Novagen). The construct used, Q1R2M, replaced arginine at position two with the starting methionine, yielding a construct for which the first glutamine has been deleted. Recombinants were expressed in BL21(DE)LysS cells, inclusion bodies prepared and protein refolded as previously described (Calvo et al. 2006). Refolded protein were concentrated, pre-cleaned on Sep-Pak C18 cartridges (Waters) and applied to a reversed phase column for purification of the recombinant proteins. Yields from 4 liters of cell culture were 330 µg for Q1R2M (330 µg). Based on reversed-phase chromatography, electrospray mass spectrometry, Edman sequencing and SDS-PAGE analysis, the recombinant protein was pure and correctly expressed (supplementary material).

2.7. Tight-binding kinetic assay

For tight-binding kinetics, monobin (10 µl at varying concentrations) and alpha-thrombin (90 µl, 100 pM final concentration) were incubated for 30 minutes at 37°C before addition of S2238 (100 µl, pre-heated to 37°C) at various concentrations, all at least 10X higher than the K_m of thrombin for S2238 under the conditions used. The reaction was then monitored as described. The K_i^* (intrinsic inhibitor constant) was calculated by fitting the data to

Morrison's equation (1) for tight-binding inhibitors (Morrison, 1969), where V_s is the steady-state velocity, V_o the uninhibited velocity, $[Et]$ the total enzyme concentration, $[It]$ the inhibitor concentration and K_i^* the intrinsic inhibitor dissociation constant.

$$V_s/V_o = \{([Et] - [It] - K_i^*) + \{([It] + K_i^* - [Et])^2 + 4K_i^*[Et]\}^{1/2}\} / 2[Et], \quad (\text{Eq. 1})$$

2.8. Slow-binding kinetic assay

To observe slow-binding visually, S2238 (100 μ l) and monobin (10 μ l) were incubated at 37°C for 30 minutes before addition of alpha-thrombin (90 μ l, 100 pM final concentration) pre-heated to 37°C. The reaction was then monitored as described for tight-binding inhibition. To derive kinetic constants from slow-binding data, monobin (10 μ l at varying concentrations similar to that used for tight-binding conditions were incubated with S2238 (100 μ l at 50, 100, 200, 300, 400 and 500 μ M) for 10 minutes at 37°C before addition of alpha-thrombin (90 μ l, 100 pM final concentration, pre-heated to 37°C). The reaction was then monitored as described. Initial and steady-state velocities were obtained by fitting the data to the equation (2) (Williams and Morrison, 1979). The equation describes the slow establishment of equilibrium between enzyme and inhibitor, where P is the measured absorbance as a defined function of the initial (V_o) and final steady-state (V_s) velocities and the apparent first-order rate constant (K_{obs}), which describes the equilibration from initial to final steady state. The initial and final steady state velocities obtained were then used to fit the data to Morrison's equation (1), to obtain apparent K_i' values. Apparent K_i' values were plotted against substrate concentration and regression analysis to zero substrate yielded the intrinsic inhibitor constant (K_i^*).

$$P = V_{st} + (V_o - V_s)(1 - e^{-K_{obs}t}) / K_{obs}, \quad (\text{Eq. 2})$$

2.9. Surface plasmon resonance analysis

Surface plasmon resonance was performed using a Biacore T-100 (Uppsala, Sweden). To detect monobin thrombin interaction, alpha-thrombin (Enzyme Research Laboratories) were diluted in sodium acetate buffer (pH 5.0) and immobilized on a CM5 sensor chip using standard amine coupling procedures. Final immobilization units were 2227 resonance units (RU). Experiments were performed at 25°C at a flow speed of 20 μ l/min. Running buffer consisted of HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% P20 surfactant). Samples were injected over a period of 300 seconds and allowed to dissociate over 1200 seconds, after which the surface was regenerated using a 30 seconds injection of 2 M $MgCl_2$. All runs were normalized by subtraction of an unmodified reference cell generated by a blank amine coupling procedure. Data obtained were fitted to several models provided in the BIAevaluation software version 3.1 (Biacore International AB). These include the Langmuir 1:1 model ($A+B \leftrightarrow AB$), heterogenous analyte model ($A1+B \leftrightarrow A1B$; $A2+B \leftrightarrow A2B$), two-state conformational change model ($A+B \leftrightarrow AB \leftrightarrow AB^*$) and bivalent analyte model ($A+B \leftrightarrow AB+B \leftrightarrow AB_2$). Of all models fitted, the bivalent analyte model gave

the overall best global fits. Rate constants for the first and second step are described by the following equations where A is the analyte (monobin) and B is the ligand (thrombin).

$$A=\text{concentration, } B[0]=R_{\text{max}}, AB[0]=0 \text{ and } AB_2[0]=0 \quad (\text{Eq. 3})$$

$$dB/dt = -[k_{a1} \times A \times B - k_{d1} \times AB] - [k_{a2} \times AB \times B - k_{d2} \times AB_2] \quad (\text{Eq. 4})$$

$$dAB/dt = -[k_{a1} \times A \times B - k_{d1} \times AB] - [k_{a2} \times AB \times B - k_{d2} \times AB_2] \quad (\text{Eq. 5})$$

$$dAB_2/dt = [k_{a2} \times AB \times B - k_{d2} \times AB_2] \quad (\text{Eq. 6})$$

$$\text{Total response} = AB + AB_2 + RI \text{ (residual bulk shift)} \quad (\text{Eq. 7})$$

To detect interaction of platelet aggregation inhibitors with the platelet fibrinogen receptor, GPIIb/IIIa (Enzyme Research Laboratories) were diluted in sodium acetate buffer (pH 5.0) and immobilized on a CM5 sensor chip using standard amine coupling procedures. Final immobilization units were ~3000 resonance units (RU). Experiments were performed at 25°C at a flow speed of 40 µl/min. Running buffer consisted of HBS-P buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% P20 surfactant). Samples were injected over a period of 500 seconds and allowed to dissociate over 5100 seconds, after which the surface was regenerated using 2 injections of 30 mM sodium acetate, pH 5.0, 2 M MgCl₂ (10 seconds at 30 µl/min). All runs were normalized by subtraction of an unmodified reference cell generated by a blank amine coupling procedure.

2.10. Bioinformatical analysis of monobin and other double-domain kunitz inhibitors

Proteins were screened against the non-redundant protein database using the BLASTP program with universal compositional score matrix adjustment against the EST database using TBLASTN (Altschul et al. 1997). Double-domain kunitz inhibitors from ticks were aligned using ClustalX (Jeanmougin et al. 1998). The alignment were manually adjusted and trimmed so that overhanging ends and the central linker that connects the two kunitz domains were removed (supplementary material). This ensured that only the most conserved parts were used in the subsequent analysis. Neighbor-joining analysis was performed using the Mega package (Kumar et al. 1994). The Poisson model for amino acid substitution was used and 10000 bootstraps were performed with complete deletion of gapped positions. Maximum parsimony analysis (10000 bootstraps) was performed using Phylip 3.65 software (Felsenstein, 1989). Maximum likelihood analysis was performed using Tree-Puzzle 5.2 (Schmidt et al. 2002). Quartet puzzling was used as tree search procedure, 10000 puzzling steps were performed with no clock-like behavior assumed. Parameters were estimated using the exact (slow) method from quartet sampling and a neighbor-joining tree. The JTT model of substitution was used and amino acid frequencies were estimated from the data set. Gamma distributed rates (using 8 Gamma rate categories) were used as model for rate heterogeneity and the Gamma distribution parameter alpha were estimated from the data set.

2.11. Apyrase activity assay

Apyrase activity was determined as previously described (Ribeiro et al. 1991; Mans et al. 1998). Briefly, 10 μ l of SGE were added to 90 μ l of substrate (2 mM ATP, ADP or AMP) with or without 5 mM $MgCl_2$ or $CaCl_2$ in 30 mM Tris-HCl, pH 7.4, 150 mM NaCl. The mixture were incubated at 37°C for 30 minutes, before the reaction was stopped by addition of 33 μ l of an ammonium molybdate:sulphuric acid (25 μ l of 2.5% ammonium molybdate, 10% sulphuric acid) and 8 μ l ascorbic acid (1%) mixture. The reaction of the ammonium-molybdate with free orthophosphate produces a blue color that was allowed to react for 10 minutes before reading at 650 nm. The amount of phosphate releases was determined by comparison to a standard phosphate curve. One enzyme unit (U) is defined as the μ mole Pi released per minute. The effect of various inhibitors was tested by including these in the reaction mixture.

2.12. Anti-platelet assays

Platelet aggregation was measured using a microplate method as previously described (Mans et al. 2002c). Briefly, 10 μ l of SGE were incubated with 90 μ l of platelet rich-plasma (PRP) for 10 minutes at room temperature with shaking on a microplate shaker. Agonist (10 μ l) was added and the suspension was shaken for 10 minutes before being read at 650 nm. Agonists used were ADP (10 μ M final concentration), collagen (2 μ g/ml final concentration), thrombin (1 U/ml final concentration) and epinephrine (10 μ M final concentration). PRP were prepared by platelet-pheresis from medication free donors at the DTM/NIH bloodbank. For aggregometer studies, assays were done as previously described (Mans et al. 1998). Briefly, SGE were added to 300 μ l PRP and incubated for 10 minutes while stirring in the aggregometer and monitoring for pre-aggregation effects. Aggregation was induced using 10 μ l of agonist. Concentrations for agonists used include ADP (20 μ M), collagen (0.1 μ g/ml), platelet-activating factor (250 nM) and ristocetin (1mg/ml) final concentrations.

2.13. Assay for complex formation with GPIIb/IIIa

Gel shift assays were performed as previously described (Mans and Neitz, 2004b). Briefly, 2 μ l of GPIIb/IIIa (20 pmoles) were incubated with inhibitor (at various concentrations described in results) for 30 minutes at room temperature. This mixture was then loaded onto a 10% NuPAGE SDS-PAGE gel and run in the presence of running buffer (NuPAGE MES SDS running buffer).

2.14. Recombinant expression of the monogrins, savignygrin and disagregin

Genes coding for savignygrin A+ (GI: 21435980) and disagregin (an optimized gene were constructed because no sequence data is available for disagregin) were chemically synthesized (GeneArt, GmbH, Germany). Both genes possessed an Nde I site followed by an in-frame methionine codon at its 5' end and a stop codon followed by an Xho I site at the 3' end. This allowed for cloning into the pIVEX 2.3d expression vector (Roche). The genes for the monogrins, MG1 (GI: 114152966) and MG2 (GI: 114152968) were obtained from a cDNA library that was constructed for the salivary glands of *A. monolakensis* and processed similarly to savignygrin and disagregin to allow their cloning into the pIVEX 2.3d

expression vector. Subcloning into the expression vectors and verification of their DNA information were performed using standard molecular biological techniques. TOP10 *E. coli* cells (Invitrogen) were used as recombinant host during all DNA manipulations. After recombinant clones with the correct inserts were identified and verified using DNA sequencing, high yields of plasmids were obtained from 50 ml culture by performing minipreps. Proteins were expressed recombinantly in an *in vitro E. coli* system (RTS500 *E. coli* Disulphide Kit, Roche), that allowed the formation of disulphide bonds. Procedures as described in the RTS 500 manual were followed for expression using 15 µg/50 µl total plasmid vector overnight at 37°C. The RTS 500 ProteoMaster device utilizes a 10000 MWCO membrane for exchange of essential components required for expression. As such, all recombinants (molecular masses > 10 kDa) accumulated in the feeding chamber of the system, while most of the components involved in cell free expression (*E. coli* transcription and translation machinery and other proteins found in the cell extracts) remained in the reaction chamber. This allowed for separation of most cellular components before purification. The cell feeding chamber solution was removed and concentrated and desalted using 5000 MWCO ultra-filtration devices (Millipore). The remaining protein was then applied to a reversed phase HPLC column (supplementary materials). Fractions were collected and tested for anti-platelet activity. For savignygrin two peaks with activity were obtained and their migration profiles on non-reducing SDS-PAGE correspond with the two conformational iso-forms previously observed for high molecular mass savignygrin (supplementary materials) (Mans et al. 2003). Mass spectrometry showed that the expected molecular masses were obtained for savignygrin, while N-terminal sequencing confirmed the identity of the constructs (supplementary materials). Inhibition of ADP-induced platelet aggregation by the recombinants gave IC₅₀ values of ~100–150 pM that corresponded well with that obtained previously for the wild-type constructs (Karczewski et al. 1994; Mans et al. 2002c). The recombinants as such, were considered to be in a pure active form that could be used for analysis.

2.15. Plasmon resonance analysis of GPIIb/IIIa interaction

3. Results

3. 1. Anti-clotting factors in the SGE of *A. monolakensis*

Crude SGE inhibited both extrinsic (APTT) and intrinsic (PTT) pathways of blood coagulation using human plasma (Fig. 1A). Recalcification time of chicken plasma was also significantly prolonged (Fig. 1B). Because it is known that soft ticks from the genus *Ornithodoros* possess fXa and thrombin inhibitors (Waxman et al. 1990; Gaspar et al. 1995; van de Locht et al. 1996), inhibition of the chromogenic activity of these coagulation factors were measured. Only thrombin activity was significantly inhibited (0.3% of a salivary gland equivalent gave 50% thrombin inhibition), while fXa activity showed no significant inhibition even at 50% of a salivary gland equivalent (Fig. 1C). In addition, no homologs to the fXa inhibitors from *Ornithodoros* ticks could be found in a cDNA libraries constructed for fed and unfed *A. monolakensis* (Mans et al. 2007), suggesting that this inhibitor or gene is absent in this species.

3.2. Purification of anti-clotting activity in *A. monolakensis*

In order to determine whether the thrombin inhibitory activity was the major activity present in the SGE, the anti-PTT, anti-APTT and anti-thrombin activities was purified. To do this, SGE (100 glands) were prepared and fractionated on an anion exchange column (Fig. 2). Inhibitory activity for the APTT and PTT tests using human plasma, as well as the hydrolysis of chromogenic substrate by thrombin eluted in a single peak with an acidic iso-electric point. Fractions that showed inhibitory activity were pooled and further fractionated using reversed phase chromatography and showed that the inhibitory activities all eluted in a single peak (Fig. 2). Mass spectrometry of this peak indicated that the dominant protein had a molecular mass of 13663.1 Da and that it is more than 90% pure (supplementary material). The protein was N-terminally blocked as determined by Edman degradation. To identify the inhibitor a peptide mass fingerprint were generated and used to screen a cDNA library constructed for the salivary glands of *A. monolakensis*. A single contig (AM-28) was identified with a significant score (143) for which 20% sequence coverage were obtained (supplementary material). This contig is representative of 5 identical full-length clones found in the cDNA library sequenced (2914 clones sequenced), with no additional closely related sequences that would suggest the presence of isoforms. This potential thrombin inhibitor was named monobin (Genbank accession number: 114152962). Final yield for monobin was 5.5 µg, which gives 0.55% of the total salivary gland protein. If the inhibitory profiles of, for example, the APTT pathway is compared between salivary gland extract and purified monobin (Fig. 1), similar prolongation times is observed between 50% salivary gland equivalent and 15 nM wild-type inhibitor. If it is assumed that monobin are responsible for the majority of inhibitory activity observed, then it can be calculated that 15 nM monobin in 1/2 salivary gland corresponds to a total of 6.55 µg monobin/100 glands (0.655% of total gland protein). This corresponds well with the final yield obtained for monobin (83% specific yield) and suggests that this inhibitor is indeed the major inhibitor of blood coagulation found in the glands.

3.3. Primary structure of monobin

The cDNA identified for monobin, codes for a protein of 144 amino acids that includes a signal peptide, indicating targeting to the secretory pathway and in the case of soft tick salivary glands, most probably to the salivary gland secretory granules that is secreted during feeding. The mature protein has 128 amino acid residues with a predicted mass of 13678 Da that differs from the mass of wild-type monobin by 15 Da. This corresponds most closely with an N-terminal modification of glutamine to pyroglutamine and can explain the fact that the wild-type protein was N-terminally blocked. The mass calculated are based on the full oxidation of its 12 cysteines to give 6 disulphide bonds and this correlates with the fact that it is a secreted protein. It's predicted iso-electric point is 4.21 and corresponds with the elution pattern observed for wild-type monobin on the anion exchange column.

3.4. Slow, tight-binding of monobin to thrombin

Wild-type monobin shows slow-binding kinetics as exhibited by the differences in slope when inhibitor is pre-incubated with thrombin or substrate before the reaction is started with substrate or thrombin (Morrison, 1982), respectively (Fig. 3A). Under tight-binding

conditions at 300 μ M of chromogenic substrate, the wild-type monobin exhibit tight-binding kinetics and fitting the data to Morrison's equation (Morrison, 1969) yields an apparent intrinsic inhibitor constant (K_i^*) of 7 pM (Fig. 3B). When kinetics were performed under slow-binding conditions, a correlation is observed between the apparent K_i' and substrate concentration, that indicate competitive inhibition. Regression to zero substrate yields an intrinsic inhibitor constant (K_i^*) that is similar to that obtained for tight-binding conditions (Fig. 3C). These results are similar to that obtained previously for the thrombin inhibitors ornithodorin and savignin, described from *Ornithodoros* species (van de Loch et al. 1996; Nienaber et al. 1999). The wild-type inhibitor was also tested for inhibitory activity against other serine proteases that included trypsin, chymotrypsin, plasmin, elastase and tryptase. In all cases no significant inhibitory activity were observed using wild-type monobin up to 500 pM (results not shown), indicating that monobin is specific for thrombin. A recombinant version of monobin (Q1R2M) showed similar kinetics to the wild-type protein (Fig. 3D–F). In addition it showed similar inhibition patterns for the APTT and PTT pathways and prolonged the recalcification time of chicken plasma (Fig. 1D–F). This confirmed that the AM-28 sequence were indeed coding for monobin.

3.5. Biacore analysis of Q1R2M and thrombin interaction

Plasmon resonance analysis showed that recombinant monobin bound in a concentration dependent manner to alpha-thrombin, confirming the results obtained with chromogenic substrates (Fig. 3G). Kinetic models expected to describe binding (1:1 binding or two-state conformational change model), did not fit the data well. The best fits that could be obtained were with the bivalent analyte and heterogeneous ligand models. The bivalent analyte model, assumes two independent binding sites in the analyte that can interact with separate ligand molecules, as is found for antibodies (Crothers and Metzger, 1972). In this model, affinity constants cannot be determined due to avidity effects. The heterogeneous model assumes two independent binding sites due to ligand heterogeneity, and fitting gives dissociation constants of K_{D1} ~400 pM and K_{D2} ~40 nM, respectively. In the case of monobin-thrombin interactions, there are two binding sites on thrombin (fibrinogen-binding exosite and active site) and two binding sites on monobin (N- and C-terminal BPTI-domains) that could mimic kinetics for either bivalent analyte or heterogeneous ligand models. In addition the slow-binding found for monobin-thrombin could further complicate fitting to the standard models. As such, the complexity of the observed kinetics warrant a more in depth investigation that does not fall within the scope of the current paper.

3.6. BLAST and phylogenetic analysis

BLAST analysis of monobin against the non-redundant database retrieves itself (e-79), ornithodorin (9e-11), savignin (8e-10) and boophilin (2e-09). These are all BPTI/Kunitz proteins that possess two BPTI-domains linked in tandem. Ornithodorin is the thrombin inhibitor from *O. moubata* and savignin its ortholog from *O. savignyi* (van de Loch et al. 1996; Nienaber et al. 1999; Mans et al. 2002b). Boophilin has been annotated as a thrombin inhibitor from the hard tick *Boophilus microplus* (Metastriate), although no data has yet been published. Analysis of the nucleotide database using TBLASTN indicates sequences with similarity to boophilin for *Ixodes scapularis* (Prostriata) and *Amblyomma cajanensis* (Metastriate). As such, it is possible that orthologs exist between hard and soft ticks, that

target thrombin. To investigate this on a more rigorous basis, phylogenetic analysis of double-kunitz domain proteins from ticks were performed, using the assumption that the double-kunitz domain have evolved once within the tick lineage. Other known double-kunitz proteins from ticks include the ixolaris homologs (that target the exosite of fXa) from *I. scapularis* and *I. pacificus* (Francischetti et al. 2002). Amblin is a thrombin inhibitor from the hemolymph of the hard tick *A. hebraeum* (Lai et al. 2004). Additional double-kunitz proteins for which no function has yet been elucidated were also found in the cDNA libraries sequenced for *A. monolakensis* (AM-20 and AM-42) and *I. scapularis* were included in the analysis. Neighbor-joining, maximum parsimony and maximum likelihood analysis all give trees that are similar in topology (Fig. 4). In all trees, monobin, ornithodorin and savignin group in a monophyletic clade with good support. Boophilin, however, does not group with these proteins, but rather with proteins from *I. scapularis* and *A. cajanense*. In addition, the thrombin inhibitor from *A. hebraeum* groups in a distinct clade from the other potential thrombin inhibitors found in hard ticks. As such, there seem to be no support for the hypothesis that double-domain kunitz inhibitors from hard ticks are orthologs of the soft tick thrombin-inhibitors, but the data do support orthology of the soft tick inhibitors.

3.7. Anti-platelet factors in the SGE of *A. monolakensis*

Crude SGE inhibited platelet aggregation induced by ADP, thrombin, epinephrine and collagen (Fig. 5A–D). Inhibition of thrombin-induced platelet aggregation could be accounted for by monobin, the thrombin inhibitor identified in crude SGE. However, it was, previously shown that apyrase (ATP-diphosphohydrolase) activity can be responsible for inhibition of ADP and collagen-induced platelet aggregation in saliva of the hard tick *I. scapularis* and the soft ticks *O. moubata* and *O. savignyi* (Ribeiro et al. 1985; Ribeiro et al. 1991; Mans et al. 1998). SGE of *A. monolakensis* showed significant levels of apyrase activity (Table 1) that hydrolyzed ATP in the presence of magnesium and ADP in the presence of magnesium or calcium. Specific activities were lower in *A. monolakensis* for all substrates tested when compared with data for *O. savignyi* (Mans et al. 1998), although similar trends in the ATP/ADP ratios were seen. The activity responded in a similar way to inhibitors as previously observed for apyrase from *O. savignyi* (Table 1) and was heat inactivated at 60°C (supplementary material). This suggests that apyrases from soft ticks have similar properties and are most probably evolutionary related.

3.8. Additional platelet aggregation inhibitors in SGE

SGE treated at 70°C still inhibited ADP-induced platelet aggregation (Fig. 5A), although slightly less than untreated extract, indicating that additional factors that inhibit platelet aggregation were present in SGE. This was also seen when aggregometer tracings of platelet aggregation induced by ADP in the presence of untreated or heat-treated SGE were compared (Fig. 5E). In the presence of untreated SGE, inhibition of platelet aggregation was characterized by initial shape change (decrease in transmittance), aggregation of platelets, followed by disaggregation. The tracing furthermore returned to an oscillating pattern that resembled that of resting platelets. These results were similar to that obtained for purified apyrase from *O. savignyi* that disaggregated platelets and induced a reversal of shape change from an activated spherical form to a discoid resting form (Mans et al. 2000). However, platelets in the presence of heat-treated SGE, undergo shape change upon addition of ADP

and aggregate to a higher extent than observed with untreated SGE, correlating with the inactivation of apyrase activity (Fig. 5E). The platelets do, however, disaggregate, indicating that other inhibitory factors in addition to apyrase activity exist in the salivary glands. In contrast to normal SGE, the tracing does not return to the oscillating pattern observed for resting platelets, indicating that although the platelets disaggregated, no reversal of shape change occurred. Such a post-activation inhibitory response that does not result in reversal of shape change is associated with inhibitors that target the fibrinogen receptor, GPIIb/IIIa (Mans et al. 2002d). Previously, work demonstrated that in addition to apyrase, GPIIb/IIIa antagonists (savignygrins and disagregins) exist in SGE of *O. savignyi* and *O. moubata*, which raises the question regarding their presence in *Argas* ticks (Karczewski et al. 1994; Mans et al. 2002c).

3.9. GPIIb/IIIa antagonists in the SGE of *A. monolakensis*

To test whether a GPIIb/IIIa antagonist exists in SGE of *A. monolakensis*, a gel shift assay was used to test for the interaction of SGE components with GPIIb/IIIa (Fig. 5F–H). This assay is based on the fact that various integrin antagonists protect the GPIIb/IIIa heterodimer against dissociation in the presence of SDS (Mans and Neitz, 2004b; Thibault, 2000; Zolotarjova et al. 2001). Initial assays found no protection against GPIIb/IIIa dissociation by SGE (Fig. 5A). In contrast, crude salivary gland extract from the soft tick *O. parkeri* showed a gel shift for GPIIb/IIIa, indicating that the assay works with crude SGE (Fig. 5B). Savignygrin homologs were also found in a cDNA library of *O. parkeri* (I. Francischetti, personal communication), which correlate with this observed function in the crude SGE. However, there may be antagonists that interact with GPIIb/IIIa but do not protect GPIIb/IIIa against SDS-induced dissociation. To test this, synthetic genes for disagregin and savignygrin were constructed and were expressed as active recombinant proteins with IC₅₀ values that corresponded to that of the wild-type proteins (supplementary material). Recombinant savignygrin protected GPIIb/IIIa against SDS-induced dissociation, while disagregin did not (Fig. 5A and 5C). Disagregin did, however, compete with savignygrin for binding to GPIIb/IIIa, thereby inhibiting the protective effect that savignygrin exhibits against SDS-induced dissociation (Fig. 5C). Using this rationale, SGE of *A. monolakensis* had a similar competitive effect as observed for disagregin (Fig. 5A). This suggested that a GPIIb/IIIa antagonist exists in SGE, but that this inhibitor does not protect the GPIIb/IIIa complex against dissociation by SDS.

3.10. Purification of anti-platelet factors from SGE of *A. monolakensis*

In order to characterize the anti-platelet aggregation activities observed in SGE of *A. monolakensis*, inhibitory activities were purified by chromatography. The same set of anion exchange fractions (AEC) previously fractionated for monobin, was assayed for anti-platelet activity (Fig. 7). Inhibitory activity against ADP-, collagen- and epinephrine-induced platelet aggregation eluted in the flow-through of the column, indicating inhibitory activity with a basic iso-electric point (Fig. 7). Inhibitory activity also eluted at a time that corresponded with apyrase activity (Fig. 7). This inhibitory activity could be inactivated at 70°C, suggesting that this activity is solely due to apyrase (results not shown). The flow-through from AEC was directly applied to a cation exchange column (CEC) under the same buffer conditions used for anion exchange chromatography. Platelet aggregation inhibitory

activity against ADP, collagen and epinephrine-induced platelet aggregation eluted in two distinct peaks labeled monogrin 1 (MG1) and monogrin 2 (MG2) (Fig. 7). These fractions were applied to a reversed phase column (RPC), where activity eluted for both fractions in a single peak. Final concentrations obtained indicate that ~15 µg was obtained for both inhibitors, respectively, giving approximate yields of 1–2% of the total SGE for each inhibitor.

3.11. Analysis and purity of monogrins

SDS-PAGE analysis indicated that purified monogrins have similar molecular masses of approximately 10 kDa (supplementary material). Mass spectrometry indicated that MG1 has a molecular mass of 9827 Da and MG2 of 9941 Da (supplementary material). Peptide mass fingerprint analysis against an *A. monolakensis* salivary gland cDNA library identified MG1 as contig AM-30A and MG2 as AM-30B (supplementary material). For both six clones were obtained in the cDNA library. The masses calculated from their sequences (MG1 – 9702.9 Da and MG2 – 9823.2 Da), differ by 124 Da and 118 Da, respectively. The differences in mass might be explained by the fact that Edman degradation indicated that both inhibitors were N-terminally blocked, indicating that some form of modification occurred to the inhibitors. Prediction of the iso-electric points indicate that MG2 is more basic than MG1, and also corresponds with their elution patterns during cation exchange chromatography. Given these results, AM-30A and AM-30B are the probable sequences that codes for MG1 and MG2, respectively. The presence of signal peptides indicates that these inhibitors will be targeted to the endoplasmic reticulum during synthesis. The absence of ER-retention signals in the sequences indicates that the proteins will be targeted through the secretory pathway to the secretory granules in the salivary glands that are secreted during feeding.

3.12. Inhibition of platelet aggregation by monogrin

Both wild-type monogrins inhibited ADP-induced platelet aggregation with an IC_{50} of ~150 nM (Fig. 8A). This is similar to other known platelet aggregation inhibitors found in ticks, leeches and snake venoms and indicates that these inhibitors will function in the correct physiological range to inhibit platelet aggregation during feeding (Wang et al. 1994; Karczewski et al. 1994; Mans et al. 2002c). The monogrins comprise approximately 4% of the total salivary gland protein. If only 50% of the salivary gland proteins are secreted during feeding and a feeding site volume of 10 µl – 50 µl are assumed, the total concentrations of the monogrins can reach between 800–4000 nM at the feeding site, which would be well within their inhibitory range and suggest that they will indeed have a significant physiological effect during feeding. The low levels of monogrins that were purified did not allow for further characterization. Thus, in order to confirm the activities and allow further characterization of the identified contigs, AM-30A (MG1) and AM-30B (MG2) were expressed recombinantly as single domain proteins in a cell free *E. coli* system. Recombinant proteins were purified using reversed phase chromatography and analysis by mass spectrometry, SDS-PAGE and Edman sequencing confirmed their identity and purity (supplementary material). IC_{50} values for recombinant MG1 and MG2 corresponded well with the wild-type inhibitors and confirmed that these were indeed the inhibitors purified (Fig. 8B). Recombinant monogrins at 2X the IC_{50} concentration (300 nM) inhibited platelet aggregation induced by ADP, collagen and platelet activating factor (PAF) using an

aggregometer (Fig. 8D). In all cases platelets were activated as exemplified by a decrease in transmittance. This signifies shape change associated with platelet activation and indicates that the monogrins target platelets at a post-activation step that is generally associated with targeting of the fibrinogen receptor, GPIIbIIIa (Mans and Neitz, 2004a). The curves furthermore, do not return to the oscillating pattern found for resting platelets, but show minimal oscillation as can be expected for a spherical population. This also correlates with the inhibitory curves observed for heat-treated SGE (Fig. 5), suggesting that the monogrins were still active in the SGE after apyrase activity was inactivated. The monogrins did not inhibit platelet aggregation induced by ristocetin an agonist that aggregates platelets independently from the fibrinogen receptor (Fig. 8D), further correlating with targeting of GPIIbIIIa.

3.13. Competition of monogrins with savignygrin for binding to GPIIbIIIa

The question is thus raised whether the monogrins target GPIIbIIIa and whether they are responsible for competition with savignygrin and GPIIbIIIa observed in the crude SGE. Both wild-type and recombinant inhibitors fail to protect GPIIbIIIa against SDS-induced dissociation (Fig. 6D and E). There is, however, concentration dependent competition with savignygrin for GPIIbIIIa binding, that results in the dissociation of GPIIbIIIa in the presence of SDS. As such, the monogrins correlate with the observed competitor found in SGE and is with apyrase, the major platelet aggregation inhibitors found in the salivary glands of *A. monolakensis*. It should be noted that in both SGE and purified inhibitors, competition with savignygrin is only partially successful, unlike disagregin that seem to compete effectively with savignygrin. This could either indicate that the monogrins and savignygrin target different sites on the integrin, or that their binding kinetics differs. To investigate the kinetics of GPIIbIIIa interaction, plasmon resonance analysis was performed. All inhibitors bind to immobilized GPIIbIIIa in a concentration dependent manner that approached saturation (Fig. 8C; supplementary material). Binding was metal-ion dependant, as it was completely abolished in the presence of EDTA (results not shown). This confirmed that the monogrins bind to GPIIbIIIa. Even so, it was not possible to fit the data to any binding models, as a loss of surface activity was observed for the immobilized GPIIbIIIa. It is, however, clear that the monogrins dissociate much faster than either savignygrin or disagregin, and could explain their inability to protect against GPIIbIIIa dissociation by SDS.

3.14. Bioinformatical analysis of the monogrins

BLASTP analysis of the monogrins retrieves the savignygrins from the non-redundant database with significant E-values (E-0.009), suggesting that they are indeed orthologs. The savignygrins, as well as disagregin was previously identified as platelet aggregation inhibitors in the soft tick's *O. savignyi* and *O. moubata* (Karczewski et al. 1994; Mans et al. 2002c). Potential orthologs were also found in a cDNA library of *O. parkeri* (Ivo Francischetti, personal communication). Multiple alignment of these proteins and disagregin, shows that the monogrins and the proteins from *O. parkeri* possess the BPTI-fold and the conserved RGD integrin recognition motif that is located at the BPTI substrate-binding presenting loop (Fig. 9). The conserved disulphide bond was absent in the sequences of *O. parkeri*, which could indicate that in these proteins this loop is more flexible. The level

of conservation is fairly low between the various inhibitors. Between the *Argas* and *Ornithodoros* genera, the percentage identity range from 21–31% and the percentage similarity from 36–49%. Between *Ornithodoros* species the identity levels range from 26–45% and the similarity levels from 40–60%. Even so, 2 copies of each gene are found in *A. monolakensis*, *O. savignyi* and *O. parkeri*. In each case the in-species copies are remarkably conserved with sequence identities of 96% (Os), 88% (Am) and 52% (Op). The paralogous genes found within a species thus show more identity to each other than do the orthologs between species. This suggests in all cases very recent gene duplication events or that concerted evolution is taking place as was previously discussed for the savignygrins (Mans et al. 2003). Maintaining multiple copies would be advantageous as this will allow higher dosage effects (necessary in regard to its function as fibrinogen receptor antagonist) (Mans et al. 2003). The high level of divergence observed between species is probably not due to host immune pressures, but rather may point to the fact that only a small portion of the total sequence and structure is involved in integrin recognition.

4. Discussion

In soft ticks, anti-clotting mechanisms have been described for the genus *Ornithodoros* and include fXa (TAP and fXaI) and thrombin (ornithodorin and savignin) inhibitors (Gaspar et al. 1995; Gaspar et al. 1996; Joubert et al. 1998; Mans et al. 2002b; Nienaber et al. 1999; van de Loch et al. 1996; Waxman et al. 1990). These inhibitors belong to the kunitz BPTI-family. Their mechanism of action is non-canonical, in that their N-terminal residues are inserted into their respective enzymes active sites instead of the usual BPTI substrate-binding presenting loop (Wei et al. 1998; van de Loch et al. 1996). These similar mechanisms were used as evidence that the fXa and thrombin inhibitors shared a common thrombin inhibitor-like ancestor and that the mechanism of action was retained after gene duplication gave rise to the fXa inhibitors (Mans et al. 2002a). In the present study, we show that monobin, a thrombin inhibitor from *Argas* ticks are orthologous to ornithodorin and savignin. In contrast, a variety of non-orthologous thrombin inhibitors have been identified in hard ticks (Horn et al. 2000; Ibrahim et al. 2001; Kazimirova et al. 2002; Iwanaga et al. 2003; Lai et al. 2004; Imamura et al. 2005; Ciprandi et al. 2006; Nakajima et al. 2006). Similarly, the monogrins described in this study is orthologous to the platelet aggregation inhibitors that target GPIIbIIIa, savignygrin and disagregin described for the genus *Ornithodoros* (Karczewski et al. 1994; Mans et al. 2002c). Except for disagregin, all soft tick platelet aggregation inhibitors possess the RGD-integrin recognition motif presented on the substrate-binding presenting loop of the kunitz/BPTI-domain. In contrast, hard tick inhibitors that antagonise GPIIbIIIa do not belong to the kunitz/BPTI-family (Wang et al. 1996; Francischetti et al. 2005; Ribeiro et al. 2006). This implies that thrombin and platelet aggregation inhibitors evolved from their respective common ancestors, within the ancestral soft tick lineage. Conversely, hard tick inhibitors evolved independently of the soft tick inhibitors, most probably exclusively within the hard tick lineage.

It has been proposed that the BPTI-like platelet aggregation inhibitors from soft ticks were derived from anti-coagulants (fXa and thrombin inhibitors described in part 1) via a gene duplication event (Mans et al. 2002a). A phylogenetic analysis of the soft tick inhibitors indicate that the platelet aggregation inhibitors group into a monophyletic clade (Fig. 9B),

supporting their status as orthologs, but also group closest to the C-terminal domains of the thrombin inhibitors, supporting the alternative hypothesis previously advanced (Fig. 9C), that suggested that the platelet aggregation inhibitors were gene duplicates from the C-terminal domain (Mans et al. 2002a). The N-terminal domain of monobin, the thrombin inhibitor from *A. monolakensis*, groups within a clade that includes the N-terminal domains of the thrombin inhibitors from *Ornithodoros* (Fig. 9B). Within this clade, the fXa inhibitors (TAP and fXa) group terminally to the N-terminal domains of the thrombin inhibitors of the *Ornithodoros* genus. Since no fXa inhibitors have been detected for *A. monolakensis*, the basal position of the *A. monolakensis* inhibitor would suggest that the fXa inhibitors duplicated after divergence of the *Argas* and *Ornithodoros* genera and that fXa inhibitory activity evolved exclusively within the genus *Ornithodoros* (Fig. 9C). In addition, no fXa activity has been detected in *O. parkeri*, a New World *Ornithodoros* species. This could then suggest that fXa activity might have evolved exclusively within the *O. moubata* complex. As such, both thrombin and platelet aggregation inhibitors evolved within the ancestral argasid tick, with the possibility that the thrombin inhibitors evolved first.

While the kunitz/BPTI anti-hemostatic proteins is found in at least three soft tick species from two different genera, the question is raised regarding the conservation of the anti-hemostatic factors throughout the soft tick family. Salivary gland extracts from the soft tick *O. parkeri* have been tested for anti-thrombin or fXa inhibitory activity. Surprisingly, no anti-thrombin or fXa activity was found even though it was expected based on the present results (results not shown). This indicates that this activity was most probably lost in this species. This might not be surprising, as gene loss is a major determinant in genome evolution. Even so, this study did show that *O. parkeri* still maintain the platelet aggregation inhibitors. As such, sampling of diverse species within the soft tick family will show how well this activity is conserved and how prevalent functional losses for salivary gland components are within ticks. Presently, we cannot say how conserved the anti-hemostatic factors will be throughout the soft tick family. It is, however, of interest that the anti-hemostatic factors have been maintained in at least three diverse soft tick species. In the case of the thrombin inhibitors, their conservation is probably linked to the blood-clotting cascade being conserved for more than 400 million years (Doolittle and Feng, 1987). Thrombin itself is highly conserved throughout the vertebrates and sequence comparison between human and chicken thrombin indicates 61% sequence identity. As such, significant cross-reactivity of tick inhibitors with thrombin from various vertebrates would not be surprising. Similarly, homologs to the mammalian fibrinogen receptor that interacted with mammalian fibrinogen have been detected in avian thrombocytes (Kunicki and Newman, 1985). It is thus likely, that RGD-mimetics that recognize avian GPIIb/IIIa will also cross-react with its mammalian homolog.

The cross-reactivity observed for anti-hemostatic factors from soft ticks for avian and mammalian targets have significant implications for the origin of blood-feeding in soft ticks as well as the adaptation of soft ticks to new vertebrate hosts. Current views on the origins of ticks place them in the Devonian (350–400 MYA; early origin) or in the Late Cretaceous (110 MYA; late origin) (Dobson and Barker, 1999; Barker and Murrell, 2004; Klompen et al. 2000). Other postulates on tick origins, fall between these extremes and range from the

Permian (250–280MYA), early Mesozoic (250 MYA) to the Early Cretaceous (145 MYA) (Hoogstraal and Aeschlimann, 1982; Balashov, 1989; Balashov, 1994; Black and Piesman, 1994; Filippova, 1977). If ticks originated in the Devonian, then it would imply that soft tick anti-hemostatics evolved before the divergence of mammals and birds for which the current estimated date split range from 312–330 MYA (Benton and Donoghue, 2007). In this case, it would mean that soft ticks were already adapted to the hemostatic system of vertebrates and that this facilitated the changes in host preference, upon divergence of mammals and birds. A later origin, ranging from the Permian to Late Cretaceous, would mean that ticks evolved blood-feeding behavior after the divergence of birds and mammals. In this case, at least one soft tick lineage would have had to switch hosts.

It has been suggested that the ancestral tick lineage would have been argasid-like, and that the ancestral soft tick lineage would have been *Argas*-like, feeding originally on avian hosts (Black and Piesman, 1994; Mans and Neitz, 2004a). This would fit with a late origin for ticks after divergence of birds and mammals. As such, the presence of functional anti-hemostatics that could efficiently target the new mammalian host's hemostatic system could have allowed soft ticks to switch hosts fairly easily, thereby increasing their chances of gaining a blood-meal. A resolution to the early or late origin scenarios described above cannot be resolved yet. However, regardless of the scenario, it should be clear that the presence of anti-hemostatic components that could function across diverse vertebrate lineages would be advantageous when parasitizing new host species. Fossil evidence indicate that the main soft tick genera diverged at least 96 million years ago (Klompen and Grimaldi, 2001). This fossil has been identified as being a member of the *Carios* genus, which place it within the *Ornithodorinae*. The postulated origin for ticks in the Late Cretaceous (110 MYA) (Klompen et al. 2000), is too close to this date to allow both divergence of hard and soft ticks, as well as divergence of soft ticks into their respective genera. If a “late” origin of ticks is feasible it would be more likely to have occurred earlier in the Permian-Late Cretaceous periods. We can thus place a lower limit on the origins of anti-hemostatic factors in soft ticks at least at 96 MYA, but probably as far back as 250–145 MYA.

5. Conclusion

The present study shows that thrombin and platelet aggregation inhibitory activity is conserved between soft tick species from the *Argas* and *Ornithodoros* genera that, based on fossil evidence diverged more than 96 MYA. This implies that blood-feeding behavior evolved once within the soft tick family and that some anti-hemostatic mechanisms has likely been maintained for more than 100 million years, even though soft ticks underwent several host shifts during this period. This is indicative of the importance of anti-hemostatic molecules in the evolution of blood-feeding behavior in ticks. It also indicates why *Argas* ticks can readily feed on mammals, thereby remaining a health risk to humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Inhibition of blood-clotting and clotting factors by *A. monolakensis*. A) Inhibition of the intrinsic (APTT) and extrinsic (PTT) coagulation pathways using human plasma and crude SGE. B) Inhibition of recalcification time of chicken plasma by crude SGE. C) Inhibition of human blood coagulation factors thrombin and fXa using chromogenic substrates and crude SGE. D) Inhibition of APTT pathway by purified wild-type and recombinant monobin. E) Inhibition of PTT pathway by purified wild-type and recombinant monobin. F) Inhibition of recalcification time by recombinant monobin. S.D. is for triplicate values.

Fig. 2.

Purification of anti-clotting factors from SGE of *A. monolakensis*. The left-hand column indicates the fractionation of SGE using anion exchange chromatography (AEC). Fractions were tested for inhibition of thrombin and the APTT and PTT pathways. The right-hand column indicates the fractionation of fractions pooled from the AEC run (17–19 min***) using reversed phase chromatography (RPC). Fractions were tested for inhibition of thrombin and the APTT and PTT pathways and a single peak was pooled (40 min*).

Fig. 3.

Slow and tight-binding kinetics for monobin and thrombin. A) Slow-binding data for alpha-thrombin and monobin are indicated. For slow-binding, thrombin (100 pM) was added to chromogenic substrate S2238 (200 μM) and inhibitor without pre-incubation (I+S+E). For tight-binding, substrate was added to thrombin pre-incubated with inhibitor for 30 minutes (I+E+S). B) Inhibition of thrombin under tight-binding conditions, using 300 μM S2238 and fitting of the data to Morrison's equation for tight-binding inhibitors to obtain the intrinsic inhibitor constant (K_i^*). C) The relationship between substrate concentration and apparent K_i' obtained from slow-binding kinetic measurements. The intrinsic inhibitor constant (K_i^*) were obtained by regression to zero substrate. S.D. is for triplicate values. DEF) Kinetic data obtained for recombinant monobin. G) Plasmon resonance analysis of the interaction of Q1R2M and alpha-thrombin. Indicated are the fit to the bivalent-analyte model as well as parameters for fits to the bivalent-analyte, heterogeneous, 1:1 binding and two-state models.

Fig. 4.

Bioinformatical analysis of monobin and related sequences. The top panel shows a multiple sequence alignment of soft tick thrombin inhibitors and potential thrombin inhibitors from hard ticks. Indicated are the two kunitz/BPTI domains with their characteristic disulphide bond patterns. Boxed are the main regions of of the N-terminal and C-terminal domains known to interact with thrombin (van de Locht et al. 1996). Shading is based on the Blosum 62 matrix. The bottom panel shows phylogenetic analysis (neighbor-joining, maximum parsimony and maximum likelihood) of double kunitz domain tick proteins. Dots indicate nodes with bootstrap (neighbor-joining, maximum parsimony) or quartet puzzling (maximum likelihood) support higher than 80%. Shaded clades indicate different groups for which anti-thrombin activity has been verified or suspected. GI numbers are included in the sequence description line.

Fig. 5.

Inhibition of platelet aggregation by SGE of *A. monolakensis*. (A–D) Inhibition of platelet aggregation induced by ADP (10 μ M final concentration), collagen (2 μ g/ml final concentration), epinephrine (10 μ M final concentration) and thrombin (1U/ml final concentration) as monitored using a microplate assay method. Salivary gland extract was heat inactivated at 70°C for 10 min. to destroy apyrase activity in all cases, except where indicated otherwise in the case of ADP-induced platelet aggregation. Error bars indicate standard deviation from the mean for triplicate values. E) Aggregometer tracings of ADP-induced platelet aggregation. Indicated are control aggregation induced by 20 μ M ADP, PRP incubated with two salivary gland equivalents before addition of ADP and PRP incubated with two salivary gland equivalents treated at 70°C for 10 minutes to inactivate apyrase activity. Arrowheads indicate the addition of ADP.

Fig. 6.

Association of monograns with GPIIb/IIIa. A) GPIIb/IIIa dissociates into GPIIb (*) and GPIIIa (**) in the presence of SDS (lane 1). In the presence of savignygrin, a GPIIb/IIIa-savignygrin complex (***) forms that is SDS-resistant (lane 2). In the presence of SGE from *A. monolakensis* (1 salivary gland equivalent) this complex dissociates approximately 50% (lane 3). In the presence of SGE without savignygrin, GPIIb/IIIa dissociates fully (lane 4). SGE without GPIIb/IIIa shows no bands that correspond to the position of dissociated GPIIb/IIIa or the complex (lane 5). B) SGE of *O. parkeri* protects GPIIb/IIIa against SDS-induced dissociation. GPIIb/IIIa in the absence of SGE (lane 1). GPIIb/IIIa pre-incubated with 2 salivary gland equivalents from *O. parkeri* (lane 2). Two salivary gland equivalents from *O. parkeri* without GPIIb/IIIa (lane 3). C) Disagregin does not protect GPIIb/IIIa against SDS-induced dissociation, but competes with savignygrin for complex formation. GPIIb/IIIa (20 pmoles) dissociates in the presence of SDS (lane 1) but forms a SDS stable complex in the presence of 30 pmole savignygrin (lane 2). Incubation of the savignygrin-GPIIb/IIIa complex in the presence of disagregin shows a concentration dependent competition for GPIIb/IIIa that results in dissociation of the GPIIb/IIIa-disagregin complex in the presence of SDS (lanes 3–8). GPIIb/IIIa (20 pmoles) was incubated with 30 pmoles savignygrin and varying concentrations of disagregin (lanes 3–8 shows 180, 90, 45, 22, 11 and 6 pmoles, respectively). D) Competition of wild-type monograns with savignygrin for GPIIb/IIIa binding. Savignygrin (30 pmoles) and GPIIb/IIIa (20 pmoles) forms an SDS-resistant complex (lane 1). Wild-type monograns at 80 pmoles does not protect GPIIb/IIIa against SDS-induced dissociation (MG1 –lane 2, MG2 – lane 6). Incubation of savignygrin and GPIIb/IIIa in the presence of monograns at 20 pmoles (lane 3,7), 40 pmoles (lane 4,8) and 80 pmoles (lanes 5,9) of MG1 and MG2, respectively results in dissociation of GPIIb/IIIa in the

presence of SDS. E) The effect of recombinant monograns on the savignygrin-GPIIbIIIa complex is shown. GPIIbIIIa (20 pmoles) in the absence of savignygrin dissociates (lane 1) but forms a SDS-resistant complex in the presence of savignygrin (30 pmoles, lane 2). Incubation of savignygrin and GPIIbIIIa with recombinant monograns (MG1 or MG2) at various concentrations, 350 pmoles (lane 3,8), 230 pmoles (lane 4,9), 115 pmoles (lane 5,10), 50 pmoles (lane 6,11) and 25 pmoles (lane 7,12) leads to concentration dependent dissociation of GPIIbIIIa.

Fig. 7.

Purification of anti-platelet factors from SGE of *A. monolakensis*. SGE were fractionated using anion exchange chromatography (AEC, left-hand column) and fractions were tested for inhibition of platelet aggregation induced by various agonists as well as for apyrase activity (indicated by dashed line in top graph). The flow-through from the AEC was applied to a cation column (CEC, center column) and fractions were assayed for inhibition of platelet aggregation induced by various agonists. The right-hand column indicates fractionation of the two main CEC fractions (fraction 13* and fraction 15**) that showed platelet aggregation inhibitory activity using reversed-phase chromatography. The asterisks indicate peaks that showed inhibitory activity.

Fig. 8.

Inhibition of platelet aggregation by the monogrins. Determination of IC₅₀ values for wild-type (A) and recombinant monogrins (B) for ADP-induced platelet aggregation. Experiments were performed in triplicate and values indicate SD. Indicated are estimated IC₅₀ values. C) Surface plasmon resonance analysis of the interaction of monogrins and GPIIb/IIIa. Indicated are binding curves for MG1, MG2, savignygrin and disagregin. Concentrations were ~1500 nM for all. The dissociation curves were fit to a single exponential decay function to estimate the dissociation rate (k_d) as indicated. D) Inhibition of platelet aggregation induced by various agonists by recombinant monogrins (300 pM) as measured using an aggregometer. Concentrations of agonists used are ADP (20 μM), collagen (0.1 μg/ml), platelet activating factor (250 nM) and ristocetin (1 mg/ml) final concentrations.

Fig. 9. Bioinformatical analysis of the monograns and kunitz/BPTI-family from soft ticks. A) Multiple alignment of platelet aggregation inhibitors from the soft tick family. Indicated are inhibitors and their accession numbers, the conserved disulphide bonds found in the BPTI-family and the RGD-motif site (***). B) Phylogenetic analysis of the kunitz/BPTI-domains from soft ticks that is involved in anti-hemostatic function. Indicated is a neighbor-joining analysis (10000 bootstraps), with black dots indicating nodes with more than 75% bootstrap support. Proteins with two-kunitz domains were broken up into their N and C-terminal domains. Proteins used in the analysis includes Savignin N/C (GI: 17225024), Ornithodorin N/C (GI: 3024318), Monobin N/C (GI: 114152962), Monogrin A/B (GI: 114152966, 114152968), Savignygrin (GI: 21435983), Disagregin (GI: 544163), fXaI (GI: 25991385) and TAP (GI: 135311). C) A model for the evolution of anti-hemostatic factors in soft ticks. Indicated is the double domain thrombin inhibitor, with gene duplication events for different domains that led to evolution of fXa and platelet aggregation inhibitory activities.

Apyrase activity in SGE of *Argas monolakensis*. A) Apyrase activity is indicated as specific activity (μ moles of Pi released per minute per mg of protein). Also included are data obtained for the soft tick, *Ornithodoros savignyi* (Os) (Mans et al. 1998). B) The effect of various inhibitors on apyrase activity. Also included are data for apyrase from *O. savignyi* (Os) (Mans et al. 1998).

Table 1

A	Apyrase activity (μ moles Pi/min/mg protein)					
	Mg ²⁺		Ca ²⁺		No ions	
	Os	Am	Os	Am	Os	Am
ATP	14.58	3.37 $\pm$ 0.07	2.45	0.12 $\pm$ 0.002	0	0.02 $\pm$ 0.002
ADP	7.8	1.66 $\pm$ 0.018	11.2	0.54 $\pm$ 0.015	2	0.002 $\pm$ 0.003
AMP	0.24	0.02 $\pm$ 0.003	0.15	0.021 $\pm$ 0.003	0.038	0.027 $\pm$ 0.004
ATP/ADP	1.87	2.03	0.22	0.233	-	-

B	Effect of inhibitors on apyrase activity (%)					
	ATP-Mg ²⁺		ADP-Mg ²⁺		ADP-Ca ²⁺	
	Os	Am	Os	Am	Os	Am
Control	100	103 $\pm$ 4.5	100	100 $\pm$ 1.77	100	101 $\pm$ 4.7
EGTA (5 mM)	100	0.7 $\pm$ 0.15	30	0.34 $\pm$ 0.068	100	6.81 $\pm$ 0.2
EDTA (5 mM)	10	33 $\pm$ 0.07	30	53 $\pm$ 0.99	5	0.14 $\pm$ 0.2
NaN ₃ (20 mM)	111	106 $\pm$ 0.44	100	99 $\pm$ 3.7	109	100 $\pm$ 1.5
Vanadate (1 mM)	102	109 $\pm$ 0.5	101	130 $\pm$ 1.9	99	122 $\pm$ 1.4
NaF (2 mM)	19	26 $\pm$ 0.09	18	4.68 $\pm$ 0.06	92	56 $\pm$ 1.2
Ouabain (5 mM)	92	118 $\pm$ 1.35	93	108 $\pm$ 0.71	90	108 $\pm$ 2.15