Savignygrin, a Platelet Aggregation Inhibitor from the Soft Tick Ornithodoros savignyi, Presents the RGD Integrin Recognition Motif on the Kunitz-BPTI Fold

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Savignygrin, a platelet aggregation inhibitor that possesses the RGD integrin recognition motif, has been purified from the soft tick Ornithodoros savignyi. Two isoforms with similar biological activities differ because of R52G and N60G in their amino acid sequences, indicating a recent gene duplication event. Platelet aggregation induced by ADP (IC_{50} 130 nM), collagen, the thrombin receptor-activating peptide, and epinephrine was inhibited, although platelets were activated and underwent a shape change. The binding of α1-CD14 (P2) to platelets, the binding of purified αIIbβ3 to fibrinogen, and the adhesion of platelets to fibrinogen was inhibited, indicating a targeting of the fibrinogen receptor. In contrast, the adhesion of osteosarcoma cells that expressed the RGD motif toward vitronectin or fibrinogen was not inhibited, indicating the specificity of savignygrin toward α1β3. Savignygrin shows sequence identity to disagregin, a platelet aggregation inhibitor from the tick Ornithodoros moubata that lacks an RGD motif. The cysteine arrangement of savignygrin is similar to that of the bovine pancreatic trypsin inhibitor family of serine protease inhibitors. A homology model based on the structure of the tick anticoagulant peptide indicates that the RGD motif is presented on the substrate-binding loop of the canonical BPTI inhibitors. However, savignygrin did not inhibit the serine proteases FXa, plasmin, thrombin, or trypsin. This is the first report of a platelet aggregation inhibitor that present the RGD motif using the Kunitz-BPTI protein fold.

Integrins are a family of adhesion receptors that propitiates cell-cell and cell-matrix interactions. Numerous physiological processes like hemostasis, fertilization, neuron-neuron interaction, and inflammation are mediated by integrins (1). The functional receptor is expressed as a transmembrane heterodimer consisting of α and β subunits. To date, 17 α and 8 β subunits have been identified and form, in various permutations, more than 20 described integrins (2). Different combinations of subunits convey specificity for ligands (collagen-α2β1, fibronectin-α5β1, laminin-α1β1, vitronectin-α5β3, and fibrinogen-α1β3), although α1β3 can also recognize fibronectin, vitronectin, von Willebrand’s factor, and prothrombin (2). Most ligands recognized by integrins contain the integrin recognition motif RGD (3). Some ligands may also contain other sequences recognized by integrins such as the dodecapeptide sequence HHLGAKQAGDV from the γ-chain of fibrinogen that binds to αIIbβ3 (4).

αIIbβ3 (GPIIbIIIa) is the major integrin of platelets and the only adhesion receptor capable of mediating platelet aggregation by the binding of fibrinogen or von Willebrand’s factor (5–7). On resting platelets, αIIbβ3 exists in an inactive conformation that binds irreversibly to the γ-chain C-terminal dodecapeptide (HHLGAKQAGDVK) of immobilized fibrinogen (5). The unactivated form also has a ligand-binding site accessible to small molecules that contain RGD, KGD, RYD, or OrnGD motifs, which are presented on mobile recognition loops protruding 14–17 Å from the protein core (6). The ligand-binding site can also be reached by RGD peptides, which extend 11–32 Å from the surface of polyacrylonitrile beads (8). These results suggest that the binding pocket in unactivated αIIbβ3 may resemble a narrow cavity buried 10–20 Å below the protein surface (6). Upon platelet activation by various agonists, αIIbβ3 undergoes a conformational change that allows the binding of macromolecules that contain the RGD motif. The ligand-binding site is discontinuous and is formed by both αIIb and β3 subunits. A receptor-bound divalent cation within the ligand-binding site interacts with aspartic acid-containing ligands, as found in the RGD motif (2, 5).

Inhibitors specific for integrins have great potential for the study of integrin function and the development of pharmaceutical compounds (7). The most extensively characterized natural inhibitors are the snake venom disintegrins that are involved in the maintenance of hemorrhage by the inhibition of platelet aggregation (9). Platelet aggregation is compromised by the inhibition of fibrinogen binding to integrin αIIbβ3, which mediates platelet-platelet interaction. Disintegrins are a family of low molecular mass (5,400–9,000 Da) proteins that contains the RGD motif except for barbourin, which contains the sequence KGD (10). Most disintegrins inhibit platelet aggregation with IC_{50} values 3,000–30,000× lower (nanomolar concentration range) than the tetrapeptide RGDS (micromolar range) because of the conformation-restricted presentation of the RGD motif (11). The three-dimensional structures of the snake venom disintegrins echistatin, kistrin, flavodrin, dendroaspin, and the leech-derived decorin have been elucidated in solution with the use of nuclear magnetic resonance techniques (12–16). Disintegrins do not possess a classical secondary structure but rather a dense core consisting of β-turns kept intact by disulfide bonds with a protruding loop region that presents the RGD motif. The sequences flanking the RGD motif as well as the spatial configuration of the RGD motif at the end of the loop...
have been found to be important contributors to specificity for different integrins (17).

Inhibitors unrelated to the disintegrin family but proposed to bind to αIIbβ3 have been described for both hard and soft ticks (18–19). The inhibitor variabili, from the hard tick *Derma-
centor variabilis*, contains a RGD motif that is not flanked by cysteine residues, making it unique in this respect (18). Dis-
agregin from the soft tick *Ornithodoros moubata* contains no RGD sequence, and it was found that the γ-fibrinogen sequence HHLGGALQAGDV competes with its binding to soluble αIIbβ3. This indicates an inhibition mechanism distinct from disinte-
grins (19–20). Inhibitors of the coagulation cascade serine pro-
teases of the host have also been described for soft ticks. These include fXa δ (TAP and fXa) and thrombin (ornithodorin and savignin) inhibitors (21–27). All possess the Kunitz bovine pancreatic trypsin inhibitor (BPTI) fold, although the mecha-
nisms of inhibition differ from those of the canonical BPTI-like inhibitors. Whereas canonical BPTI-inhibitors present a sub-
strate-binding loop to the active site of their respective en-
zymes, the tick inhibitors insert their N-terminal sequences into the active site (21–28). This study describes the platelet aggregation inhibitor savignygrin, from the soft tick *Orni-
thodoros savignyi*, that presents its RGD motif on the sub-
strate-binding presenting loop of the canonical BPTI inhibitors. The presence of the RGD motif in the BPTI fold presents a new protein fold that can be manipulated for investigations into integrin structure/function relationships.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were of analytical grade, and double-
distilled deionized water was used in all experiments. ADP (dimonomethyl-
hexylammonium) salt, DTT, fibrinogen, 4-vinylpyridine, and thrombin
receptor-activating peptide (TRAP) were obtained from Sigma. Guani-
dinium chloride was obtained from Merck. Collagen and epinephrine were from Diagnostica Stago. α-CD41, P2-FITC was purchased from Immunotech (Beckman Coulter). Human αIIbβ3, α-thrombin, activated fXa, and plasmin were obtained from Enzyme Research Laboratories (South Bend, IN). Chromozym TH, Chromozym X, Chromozym PL, bovine trypsin, and *N*-α-benzoyl-
arginyl-p-nitroanilide were purchased from Roche Molecular Biochemicals.

**Platelet Aggregation Assays**—Platelet aggregation studies using an aggregometer were performed as described to monitor the inhibition of platelet aggregation induced by ADP (10 μM), collagen (40 μg/ml), TRAP (50 μM), and epinephrine (10 μM) (29). A microplate assay was used for the platelet aggregation activity during purification and for IC₅₀ determinations of ADP-induced platelet aggregation (30). For the temperature stability assay, savignygrins (14 μg of protein in 300 μl of 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4) were incubated at 94°C for different time periods and immediately placed on ice for 10 min before centrifugation (14,000 × g for 15 min at room temperature). Shape change and degranulation of platelets was assessed by scanning and transmission electron microscopy (31).

**Purification of Inhibitors**—Salivary gland extracts (40 salivary glands, 1,600 μg) were prepared by sonification (29). Size exclusion high performance liquid chromatography (SEHPLC) was performed under isostructural conditions (20 mM Tris-HCl, 0.15 M NaCl, pH 7.6), and a linear gradient (0–60% 1 M NaCl over 28 min) was used for anion exchange high performance liquid chromatography (AEHPLC) (32). Fractions from AEHPLC were desalted and fractionated using reverse phase high performance liquid chromatography (RP-HPLC) with a linear gradient (0–100% buffer B, 60 min) of buffer A (0.1% trifluoroacetic acid, 0.1% acetonitrile) and buffer B (0.1% trifluoroacetic acid, 60% acetonitrile) (33). Collected fractions were dried in a vacuum concentrator (Bachoffer), rechromatographed with AEHPLC, and desalted with RP-HPLC.

**Amino Acid Analysis and N-terminal Sequence Determination**—Pro-
teins were quantified using amino acid analysis; free cysteine residues were alkylated by performic acid oxidation, and thrombin by hy-
drosis with methanesulfonic acid (34–35). To determine whether all cysteine residues are present in disulfide bonds, an inhibitor (250 pmol) was denatured with 8 M guanidinium chloride for 2 h in the presence or absence of 4% DTT and then alkylated with 4-vinylpyridine. Alkylated protein was desalted using RP-HPLC and vacuum-dried be-
fore amino acid analysis, activity measurements, and N-terminal se-
quence determination (36). N-terminal amino acid sequencing (1 nmol of protein) was performed with a gas phase amino acid sequencer (37).

**Mass Spectrometry and Peptide Mass Fingerprinting**—The molecular masses of the native or alkylated inhibitors were determined by elec-
trospray mass spectrometry (ESMS) (21). Peptide mapping was per-
fomed using trypsin digestion, and subsequent analysis was accom-
plished by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (33).

**Cloning and Sequencing of the High Molecular Mass (+) Form of Savignygrin**—The cloning strategy used for savignin was followed (27). Single strand cDNA was synthesized from total RNA using a poly(T) anchor primer (GCT ATC ATT ACC ACA ACA CTC T(3)Y) (50 bp). Full-
length double-stranded cDNA was synthesized using single strand cDNA and the Marathon cDNA amplification kit (CLONTECH) accord-
ing to the manufacturer’s instructions. The cDNA gene and the 3′-untranslated region (3′-UTR), a degenerate primer (TAYCARC-
CNGARTGGTYTIG) was designed from the first seven amino acids (YQFPECLE) obtained by Edman degradation and used with the anchor primer to amplify a 300-bp product. To obtain the 5′-UTR and signal peptide sequence, a gene-specific primer (GSP, CCGATTTCGCGCATC-
A CCTT) complementary to the coding sequence of the last six amino acids of savignycin (KKAAGNA) was designed. A 310-bp product was amplified from full-length cDNA using the gene-specific primer and the AP2 adapter primer (CLONTECH). The products of at least three PCR reactions were cloned, and at least three different clones of each product were sequenced from both up and downstream ends.

**Cloning and Sequencing of the Low Molecular Mass (−) Form of Savignygrin**—3′-RACE with the degenerate primer yielded only a high mass inhibitor. A clone obtained from the 5′-RACE gave a sequence that differed at a single nucleotide, giving a R52G difference in the translated amino acid sequence. To determine whether this might be the low mass form, a primer (GrinAB, ACTATTTCCGCTCTGAAG) was designed and used in a 3′-UTR RACE reaction which gave a 170-bp product that showed both R52G and N60G differences with the high mass inhibitor sequence. To con-
firm this difference, a primer (LMM, TGTACCTCTCCTTGAA) was used in a 3′-UTR RACE reaction which gave a sequence with both differences observed with the high mass inhibitor sequence. 5′-RACE was performed as above and gave a 400-bp product that completed the full length low mass inhibitor sequence.

**Inhibition of the Binding of Monoclonal Antibody P2 (α-CD41) to Platelets by Savignygrin**—Monoclonal antibody P2 has been shown to interact specifically with αIIbβ3 (CD41) of the intact αIIbβ3 complex (38). The inhibition of P2 binding to platelets was assayed using flow cytometry (39). Platelet-poor plasma was prepared (31) and used to dilute platelet-rich plasma to a count of 300 × 10⁶ platelets/liter. Platelet-rich plasma (20 μl), savignyrin (20 μl at various concentrations), and 150 μl of Tyrode solution (0.4 mM NaH₂PO₄, 0.4 mM NaCl, 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO₃, 0.5 mM CaCl₂, pH 7.4, 3.5 mM/ml bovine serum albumin) were incubated for 30 min at room temperature without stirring in the presence or absence of ADP (20 μM final concentration). P2-FITC-conjugated monoclonal antibodies (10 μM) were added and incubated for 30 min. The unfixed platelet solution (120,000 plate-
lets/10 μl) was diluted to 500 μl with Tyrode solution before analysis with an Epics 2-flow cytometer (Coulter Electronics, Inc.). Fluorescent measurement (10,000 events) was gated to count intact, non-aggregated platelets (3–5-μm diameter). Three experiments were used to deter-
mine the mean fluorescence.

**Fibrinogen/αIIbβ3 Enzyme-Linked Immunosorbent Assay (ELISA)**—The inhibition of the binding of purified αIIbβ3 to immobilized fibrinogen was performed as described (40). αIIbβ3 was detected with P2-FITC (50× dilution of stock) using a Fluorescan Ascent FL (Thermo Lab-
systems) fluorometer.

**Integrin Specificity of Savignygrin**—The specificity of the savignygrins for other integrins was investigated using the osteosarcoma cell line MG-63, which is known to express the integrin αvβ3 and is able to

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1 The abbreviations used are: fXa, coagulation factor Xa; TAP, tick anticoagulant peptide; BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; TRAP, thrombin receptor-activating peptide; FITC, fluo-
rescein isothiocyanate; SEHPLC, size exclusion high performance liquid chromatography; AEHPLC, reversed phase high performance liquid chromatography; ESMS, electrospray mass spectrometry; MALDI-
TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; UTR, untranslated region; RACE, rapid amplification of cDNA ends; RMSD, root mean square deviation.
bind to vitronectin and fibrinogen (41). Adhesion studies were performed as described (18). The adhesion of platelets to fibrinogen was also investigated as described (19).

**Protein Fold Prediction of Savignygrin and Disagregin**—The amino acid sequences of disagregin and savignygrin were analyzed using the EMBL advanced WU-BLAST 2.08 server (BLASTP2) with the non-redundant data base (nrdb95), using the default settings (42). Protein fold prediction was performed by submission to the three-dimensional PSSM Server (43). Protein family classification was performed with the Family Pairwise Search version 2.0 (44). Multiple sequence alignment was performed with ClustalX, using the PAM250 matrix and default gap penalty options (45).

**Modeling of Savignygrin on the Structure of TAP**—The structure of savignygrin and disagregin were modeled using the NMR structure of TAP (PDB code 1TAP) (46) and the MODELLER (47) package. Root mean square deviation (RMSD) values between structure pairs were determined by fitting of the backbone structures using the McLaughlan algorithm (48) as implemented in the protein least squares fitting program ProFit V1.8 (www.biochem.ucl.ac.uk/~martin/#profit). The quality of the modeled structures was assessed by construction of Ramachandran plots using Procheck (49). The structure of TAP was obtained from the Research Collaboratory for Standard Bioinformatics Protein Data Bank (www.rcsb.org/pdb/) (50). All worm figures and surface models were constructed with the Graphical Representation and Analysis of Surface Properties (GRASP) program (51).

**Assay for Serine Protease Inhibitory Activity**—Serine protease inhibitory activity was assayed as described (21). The concentrations used were 2.6 μM savignygrin (final concentration), 0.5 mM Zn, 10 mM plasmin, 50 mM trypsin, and 5 mM thrombin. All experiments were performed in triplicate.

**RESULTS**

**Purification of Savignygrin**—The inhibition of ADP-induced platelet aggregation was used as a measure of activity during purification. During SEHPLC, inhibition of platelet aggregation was observed across the whole protein spectrum (Fig. 1a) and could be ascribed to the presence of apyrase activity in the high molecular mass region (500–20 kDa) (32). Heat inactivation of apyrase (60 °C, 10 min) shifted the inhibition pattern to the low molecular mass region (<20 kDa). AEHPLC of SEHPLC fractions indicated inhibitory activity over a broad pH range (5–6) from 10–15 min (Fig. 1b). RPHPLC separated the AEHPLC fractions into two distinct peaks designated A and B (Fig. 1c). ESMS analysis showed that both peaks contained a high and a low molecular mass species. Separation of these species was performed with AEHPLC chromatography (Fig 1d). Both peaks A and B were separated into two peaks, indicating two species that differ in charge with approximate isoelectric points of 5.9 and 5.5, respectively. These peaks were designated A+, A-, B+, and B- based on charge (as observed on AEHPLC) and hydrophobicity (as observed on RPHPLC). These different species were then desalted using RPHPLC (results not shown). The yields obtained for three different purifications were 45 ± 15 μg, 37 ± 9 μg, 27 ± 9 μg, and 27 ± 9 μg for the different forms (A+, A-, B+ and B-), respectively. This corresponds to 1–3% of the total salivary gland protein for each inhibitor.

**Electrospray Mass Spectrometry of the Savignygrins**—The (+) forms have similar molecular masses (6966 Da) (Fig. 1e), whereas the (−) forms also have similar masses (6808 Da) but are 158 Da smaller (Fig. 1f). These masses correlate well with those obtained by tricine SDS-PAGE under reducing conditions (results not shown). The spectra show the M5+ to M8+ ion species for all four isoforms, which correspond well with amino acid analysis and sequence data that indicate nine lysine and arginine residues.

**Analysis of the Deduced Amino Acid Sequence of Savignygrin**—N-terminal amino acid sequence determination of the isoforms shows that they all have the same sequence and contain an RGD motif corresponding to the RED sequence of disagregin (19). The cDNA sequences for both (+)− forms correspond to the mature protein of 82 amino acids, whereas the mature chain consists of 61 amino acids with the first 21 amino acids corresponding to that obtained with Edman degradation (Fig. 2). Analysis of the immature protein using SignalP predicted the presence of a signal peptide (21 amino acids) and the correct cleavage site (52). There are relatively few differences between the (+)− forms at sequence level. The only gap present is in the 5’-UTR of the (−) form, and most differences occur at isolated positions in the 3’-UTR. Two non-syn-
The primers used during RACE and the N-terminal sequence used for degenerate primer design are shown in boldface. The stop codon (TGA), polyadenylation signal (AATAAA), and the poly(A) tail are boxed. Gray boxes indicate sequence differences between the (+/−) forms. The signal peptide and N-terminal sequences obtained with Edman degradation are underlined with a broken and a solid line, respectively.

Comparison of Data Obtained from the Deduced Amino Acid Sequences and Data from Native Savignygrins—Amino acid compositions obtained for the native inhibitors and the compositions calculated from the deduced amino acid sequence were similar. For the native inhibitors no isoleucine or valine was detected, not even after time hydrolysis of up to 72 h. Performic acid oxidation, alkylation of cysteines with 4-vinylpyridine, and ESMS of the alkylated forms confirmed that all cysteines are present. By using 4-vinylpyridine in the presence or absence of DTT, the presence of any free sulphhydril groups can be detected. No free alkylated cysteines were detected in the absence of DTT. This indicated that all the cysteines are involved in disulfide bonds (results not shown). Alkylation with 4-vinylpyridine in the presence of DTT alone abolished inhibitory activity completely (results not shown). Peptide maps were similar for the isoforms and could not account for the mass difference observed between the (+/−) forms (results not shown). The theoretical peptide masses obtained for a tryptic digest of the deduced amino acid sequence correlated well with empirical data determined for the native inhibitors. Taken together, these results indicate the target- 
v binding of α1bβ3 to immobilized fibrinogen was tested. The binding of P2-FITC to platelets was inhibited in a concentration-dependent manner both in the presence and absence of ADP (IC50, ~12 μM) (Fig. 4a). Furthermore, adhesion of α1bβ3 to fibrinogen was inhibited with an IC50 of ~3 nM (Fig. 4b). This is comparable with that of variabilin (9 nM) (18) and decorin (1.5 nM) (40). Taken together, these results indicate the target- 

Integrin Specificity of Savignygrin—α1β3 recognizes most ligands such as vitronectin and fibrinogen that bind to α1bβ3 (2). No inhibition of osteosarcoma cell adhesion to vitronectin or fibrinogen was observed during this study, not even at a concentration of 10 μM (Fig. 4c), although adhesion of platelets to fibrinogen was inhibited at concentrations of 20 nM (5% control) (Fig. 4d). This suggests that savignygrin is specific for α1bβ3.

Protein Fold Prediction for the Platelet Aggregation Inhibitors—BLAST analysis of the protein sequence of savignygrin indicated significant similarity (E-value, 4 × 10−13) to the platelet aggregation inhibitor disagregin from the related soft tick O. moubata (19). BLAST P2 analysis of disagregin and savignygrin indicated a similarity to proteins from the BPTI family with P(N) values ranging from 0.011 to 0.206 for the first 50 hits. The highest scoring protein fold founds for disagregin and savignygrin with the three-dimensional PSSM Server are part of the BPTI-like superfamly (E-values, 0.178–0.855 for the first ten proteins) that contains the functionally diverse proteins BPTI, dentrotoxin, bungarotoxin, and knottins. Assignment to a protein family in the SCOP data base (53) using the Family Pairwise Search indicated identity to the SCOP BPTI-like superfamly with E-values of 1.6e-15 and 7.9e-16 for disagregin and savignygrin, respectively. The second highest hits gave E-values ranging from 0.09–1.42, indicating the high similarity to the BPTI protein fold relative to other protein folds.

Alignment of Savignygrin and Disagregin with BPTI Inhibitors—Alignment using the BLOSUM 45 matrix indicates a 45% identity and a 60–62% similarity between disagregin and the savignygrins (Fig. 5). Identities and similarity between the platelet aggregation inhibitors TAP and BPTI range between 16–29% identity and 30–42% similarity. The platelet aggregation inhibitors possess the conserved cysteine pattern characteristic of the BPTI fold. Like BPTI, the platelet aggregation inhibitors lack an insert that is present in the structure of TAP.
between the first two cysteines. However, the platelet aggregation inhibitors and TAP share more sequence topology than BPTI in that they have a 3-residue insertion after the P1, P1′/H11032, and P2′/H11032 position of the canonical substrate-binding loop of BPTI. This probably enlarges the loop area in the structure of the platelet aggregation inhibitors. The platelet aggregation inhibitors and TAP also lack a 2-residue insert before the fourth cysteine that is present in BPTI but have a 3-residue insert after this cysteine. Secondary structure prediction for the platelet aggregation inhibitors is similar to that of the NMR structure of TAP and shows that the R52G difference of the savignygrin isoforms occurs in the C-terminal H9251-helix of the BPTI fold.

Serine Protease Inhibitory Activity—The savignygrins have an arginine residue at the P1 position of the canonical BPTI-like inhibitors. Because inhibitors with an arginine or a lysine at the P1 position inhibit trypsin-like enzymes (28), the inhibition of trypsin, thrombin, fXa, and plasmin were investigated. No significant inhibitory activity for the different proteases was observed compared with controls. Values obtained for thrombin, fXa, trypsin, and plasmin were 114 ± 5%, 107 ± 13%, 95 ± 12%, and 105 ± 11% of the control values, respectively.

Homology Modeling of Savignygrin—Models of the structures of disagregin and savignygrin fitted to that of TAP gave RMSD values of 1.5 Å and 1.0 Å, respectively (Fig. 6a). Interestingly, the modeled structures fitted to each other gave an RMSD of 0.75 Å. Ramachandran plots showed that 6.2% of the amino acids of savignygrin were in disallowed regions (Fig. 6b). At least one disallowed residue (Cys-39) in the structure of TAP is also in the disallowed region for savignygrin (Cys-38). This is probably the reason for the second disallowed residue (Cys-13),
which is the corresponding disulfide-bonding partner. The other reason for this distortion in conformation is the presence of a 2-residue deletion in the sequence of savignygrin before Cys-13, which probably puts a torsion stress on the formation of the loop and the disulfide bond leading to the distortion of Asp-16, which also resides on this loop. The modeled structures indicate that the RGDED motif of savignygrin is located on the substrate-binding loop associated with canonical Kunitz inhibitors (Fig. 6c). The model structure also indicates the formation of three disulfide bonds (Cys5-Cys36, Cys13-Cys36, Cys32-Cys54) that correspond with those of the general Kunitz-BPTI fold. A surface model of savignygrin indicates that the RG motif extends into the surrounding solvent and forms a binding epitope with the downstream acidic residues (Fig. 6d). This suggests that the RGD motif as well as surrounding residues might indeed be involved in the inhibitory activities of these platelet aggregation inhibitors.

**DISCUSSION**

Control of the hemostatic system of the host is essential for successful tick feeding. Efficient inhibitors of the hemostatic system are thus an important part of the feeding strategy of ticks (22). Soft ticks are a rich source of inhibitors of both blood coagulation and platelet aggregation. Inhibitors of the blood clotting cascade serine proteases IXa and thrombin have been characterized (21–27). Apyrase, which inhibits platelet aggregation by the hydrolysis of ADP, has also been investigated (29, 31–32, 54). Collagen-specific platelet aggregation inhibitors and αIIbβ3 agonists have been described (19–20, 55–56). This study describes savignygrin, a potent platelet aggregation inhibitor, that is thought to inhibit platelets by targeting the platelet integrin αIIbβ3. This is the first described platelet aggregation inhibitor from soft ticks with an RGD integrin recognition motif that is presented on the canonical substrate-binding presenting loop of the Kunitz-BPTI fold.

The different isoforms from *O. savignyi* were detected in 20 individual ticks analyzed, which indicate that the (+/-) forms are gene duplicates and not allelic variants (results not shown). Interestingly, no isoforms were observed for disagregin, and no difference could be observed in the electrophoretic mobilities under reduced and non-reduced conditions (19). This suggests that the (+/-) gene duplication is a fairly recent event that occurred after the divergence of these two tick species from a common ancestor. The presence of the A/B conformational isoforms is more problematic, as no sequences were obtained that could explain the differences. However, it is not uncommon to find separation of a single protein into two peaks during RPHPCL. Such 2-peak separations are generally observed for proteins that are stable under reversed-phase chromatography conditions (low pH and high concentrations of organic mobile phase) so that native as well as unfolded forms are present (57).

Biological activity is not affected by sequence or conformational differences as indicated by similar IC50 values (~130 nM).
Savignygrin Presents RGD on the BPTI Fold

for all four isoforms. This could be accounted for in part by the RGD motif that is present in all four isoforms. Platelets are activated by various agonists via specific receptors and distinct pathways culminating in the activation of αIIbβ3, which mediates aggregation through the binding of fibrinogen (6). Platelet activation is accompanied by a shape change from a discoid (resting) to a spherical form (activated), the extension of pseudopods, and the aggregation-independent release of platelet granules (58). The activation of platelets prerinuculated with savignygrin was indicated by a decrease in transmittance during the inhibition of platelet aggregation induced by various agonists. Electron microscopic analysis of platelets incubated with savignygrin before activation with ADP confirmed the discoid to spherical shape change associated with activation (59). This suggests postactivation inhibition by the savignygrins and implicates the common denominator of platelet aggregation, the integrin αIIbβ3. The inhibition of the binding of α-CD41-FITC in the presence or absence of ADP strongly suggests that savignygrins bind to resting as well as to activated αIIbβ3. The targeting of αIIbβ3 was further supported by the inhibition of αIIbβ3 binding to fibrinogen. The discrimination observed for savignygrin between αIIbβ3 and αIIbβ2 was also found for disagregin (19). This could indicate that BPTI-like inhibitors of platelet aggregation are integrin specific. The BPTI fold might thus be useful to design integrin-specific antagonists.

It was shown that disagregin inhibits the binding of echistatin (which contains the RGD motif) to platelets, suggesting an interaction with the RGD-binding site (19). This is strongly supported by the presence of an RGD motif in the sequence of savignygrin. Although disagregin has a RED motif, and peptide studies showed that the peptide REDV does not inhibit platelet aggregation, a restricted conformation induced by the three-dimensional structure of disagregin could account for inhibitory activity (60). This is supported by the dependence of savignygrin on intact disulfide bonds for the maintenance of an active but restricted biological conformation. BPTI has also been shown to occur as a very disordered polymer in its reduced environment independently.

New strategies to investigate integrin-ligand interaction are essential to gain a comprehensive view of αIIbβ3 antagonism. This would be useful in the design of a new generation of antithrombotic therapies (7). Savignygrin and related BPTI-like inhibitors present a new protein fold that can be used in integrin-ligand investigations. The presentation of RGD on the BPTI fold could be useful to engineer novel scaffolds that exhibit both blood coagulation and platelet aggregation inhibitory capabilities.

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REFERENCES

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