Variabilin, a Novel RGD-containing Antagonist of Glycoprotein IIb-IIIa and Platelet Aggregation Inhibitor from the Hard Tick Dermacentor variabilis*

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A novel inhibitor of human platelet aggregation, named variabilin, was isolated from salivary glands of the hard tick Dermacentor variabilis using a combination of gel filtration and high pressure liquid chromatography. Variabilin was a potent antagonist of the fibrinogen receptor glycoprotein IIb-IIIa (GPIIb-IIIa; α<sub>IIb</sub>β<sub>3</sub>) and the vitronectin receptor α<sub>5</sub>β<sub>3</sub>. Amino acid sequence analysis by Edman degradation revealed that it has 47 residues, with a molecular weight of 4968.5. Like many other naturally occurring antagonists of GPIIb-IIIa, variabilin contains the RGD (Arg-Gly-Asp) motif. However, unlike the RGD-containing antagonists of GPIIb-IIIa, the RGD sequence of variabilin is not positioned in a loop bracketed by cysteine residues. It has little sequence homology to the other known naturally occurring antagonists of GPIIb-IIIa, including the disintegrins from snakes, decorin and ornatin from leeches, and disagregin from soft ticks. Variabilin is the first RGD-containing antagonist isolated from ticks.

Platelet aggregation can be mediated by the binding of either fibrinogen (Fg) or von Willebrand factor to the platelet membrane receptor, GPIIb-IIIa (1–4). In either case, it is thought that the aggregation results from the cross-linking of platelets by the multivalent binding of one of these ligands to activated GPIIb-IIIa molecules on adjacent platelets. This view is supported by the fact that certain monoclonal antibodies against GPIIb-IIIa can inhibit platelet aggregation by preventing the binding of these ligands to this platelet receptor (5, 6). Thus, the blocking of the ligand binding function of GPIIb-IIIa has become one of the approaches used clinically to prevent platelet aggregation and thrombosis (7).

GPIIb-IIIa is the most abundant platelet cell-surface protein (8). Also named α<sub>IIb</sub>β<sub>3</sub> (9), it is a member of the integrin family of receptors and serves as receptor for at least four plasma protein ligands: Fg, fibrinectin, vitronectin, and von Willebrand factor. These ligands all contain the motif RGD (Arg-Gly-Asp) (9). Upon activation of the platelets, the ligands fibronectin, von Willebrand factor, and vitronectin (Vn) appear to bind to GPIIb-IIIa via their RGD motif (10–12). In contrast, the RGD motif of Fg does not appear to be essential for the binding of Fg to GPIIb-IIIa (13). Apparently, Fg binds to GPIIb-IIIa in an RGD-independent manner via the carboxyl-terminal sequence HHLLGAKQAGDV of its γ chain (13–15). Interestingly, the binding of peptides containing RGD on the one hand, and HHLLGAKQAGDV on the other, to GPIIb-IIIa is mutually exclusive (16). It is not known if these two binding motifs bind to different sites on GPIIb-IIIa, but a variety of evidence supports this view (17, 18). Despite the possibility that the RGD motifs of Fg are not required to mediate the binding of Fg to GPIIb-IIIa, RGD-containing peptides are potent inhibitors of the binding of Fg to platelets (19).

Nature has used the ability of the RGD motif to inhibit the binding of Fg and other proteins to GPIIb-IIIa as the basis of action for a variety of naturally occurring anti-platelet agents. For example, decorin (20) and ornatin (21) have been isolated from leeches and the disintegrins (22–24) from snakes. Decorin, ornatin, and most disintegrins contain an RGD sequence. Yet some disintegrins have alternative amino acid residues instead of this sequence (23). Disagregin is a potent GPIIb-IIIa antagonist that does not have the RGD sequence (25). Changes in the RGD sequence affect the binding affinity and/or specificity of these proteins (26). Here, we report the isolation and characterization of variabilin, a novel inhibitor of platelet aggregation that is a potent RGD-containing, structurally unique antagonist of GPIIb-IIIa from the hard tick Dermacentor variabilis.

EXPERIMENTAL PROCEDURES

Materials—Adult D. variabilis (Say) were obtained from a colony maintained at Oklahoma State University. Protease inhibitors E-64, pepstatin, chymostatin, leupeptin, and phenylmethylsulfonyl fluoride were purchased from Calbiochem. BioGel P-10 was from Bio-Rad. The platelet aggregometer was from Chrono-Log Corp., Havertown, PA. The HPLC system was from Beckman Instruments. Purified human Fg was a gift from Dr. David Amrani (15). Vn was donated by Dr. Deane Mosher, University of Wisconsin, Milwaukee, WI. The monoclonal antibody AP3 was from Dr. Peter Newman (27).

Preparation of Tick Salivary Gland Extract—Undef. D. variabilis ticks (150 female and 150 male) were placed in a chamber fastened to the shaved back of a naive female New Zealand White rabbit. The ticks were allowed to feed on the rabbit for 5–6 days. Mating with male ticks enhances the development of the salivary glands in female ticks. Female ticks weighing 200–350 mg were manually detached from the rabbit and used for extraction. The salivary glands were removed from those female ticks by dissection. Crude salivary gland extract was prepared according to Karczewski et al. (25). Briefly, salivary glands were placed in 10 ml of ice-cold tick extraction buffer (TEB), which contains 20 mM Bis-Tris, pH 7.0, 0.1 M NaCl, 50 μM pepstatin, 50 μM leupeptin, 10 μM E-64, and 50 μM phenylmethylsulfonyl fluoride. Sali-
Vary glands were homogenized in a mortar kept on ice and then centrifuged in the cold at approximately 12,000 × g for 20 min. The supernatant material was transferred to a new test tube. Pellets were suspended in about 2 ml of TEB and centrifuged again. The supernatant fractions were mixed together. The mixture was made 40% acetonitrile and 0.1% trifluoroacetic acid. The solution was stirred at 4°C for 20 min and then spun at 12,000 × g for 20 min. The supernatant material was lyophilized and reconstituted in 1 ml of acetylated filtered water before use.

Purification of the Platelet Aggregation Inhibitor—A column (50 × 1.5 cm, inside diameter) was filled with BioGel P-10. The resin has an exclusion limit of 20,000 daltons. The column was equilibrated with 50 mM ammonium acetate, pH 7.0. The tick salivary gland extract (1 ml) was loaded on the column and washed with 50 mM ammonium acetate buffer (pH 7.0). Absorbance was monitored at 214 nm. Individual fractions were lyophilized, reconstituted in 200 μl of acetylated filtered water, and tested for inhibitory effect on ADP-induced platelet aggregation. The most inhibitory fractions were pooled, lyophilized, reconstituted in 100 μl of water, and applied to an HPLC ODS-DABS column (Blackmorn Instruments) equilibrated with 0.1% trifluoroacetic acid. A gradient of 0–40% acetonitrile containing 0.1% trifluoroacetic acid was used to elute bound proteins. The gradient was completed within 80 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected, lyophilized, and reconstituted in 1 ml of 1% acetic acid for 20 min. Subsequently, the plate was washed four times with 100 mM acetic acid. A gradient of 0–40% acetonitrile containing 0.1% trifluoroacetic acid was used to elute bound proteins. The gradient was completed within 80 min at a flow rate of 1 ml/min. Fractions of 1 ml each were collected, lyophilized, and reconstituted in 0.1 ml of acetylated filtered water and then tested in aggregation assay using ADP as an agonist. The test sample (20 μl) was mixed with 0.4 ml of PRP for 2 min prior to stimulation. The extent of inhibition was quantitated based on comparison of light transmittance gain in the aggregation assay of control and the tick sample.

with 4 ml of a trypsin solution (2.5 g/liter of Dubesco's phosphate-buffered saline without CaCl2) for 4 min at 37°C, neutralized with medium A, and transferred to a centrifuge tube, and the cells were removed from suspension by gentle centrifugation for 5 min. The cells were suspended in medium A and incubated at 37°C for 30 min to recover from trypsinization. After recovery, cells were washed and suspended in serum-free medium B (RPMI 1640 supplemented with 2 mM L-glutamine and 15 mg/liter gentamicin). Cell density was adjusted to 2 × 107/ml. The cells were incubated with a sample of the tick salivary gland extract at 37°C for 30 min. The cell suspension containing the tick sample was transferred to wells (100 μl/well) of a Dynatech Immulon plate that had been coated overnight at 4°C with 100 μg/ml of vonwetricin (1 μg/ml). After 2 h of incubation at 37°C, wells were washed three times with Dubesco's phosphate-buffered saline containing CaCl2 (from Sigma). Adherent cells were fixed with 100% methanol for 45 min, then washed three times with Dubesco's phosphate-buffered saline containing CaCl2, and stained with 0.1% bromphenol blue in 1% acetic acid for 1 h. After three washes with 1% acetic acid and air drying, Tris-HCl buffer, pH 9.2, was added (100 μl/well). Absorbance was measured at 595 nm using an enzyme-linked immunosorbent assay reader.

Purification of GPIb-IIa—CPIb-IIa was purified from tick salivary glands of the hard tick D. variabilis by a combination of gel filtration and reverse-phase HPLC. Crude salivary gland extract was prepared as described under “Experimental Procedures.” The extract was applied to a BioGel P-10 gel filtration column to 40 mM Tris-HCl, 150 mM NaCl, 0.5% bovine serum albumin, and 0.1% glucose. Adhesion of resting platelets (RPs) and non-exogenously stimulated platelets (NPs) was studied. For RP adhesion, prostaglandin E1 at 1 μg/ml was added to the PRP. For adhesion of NPs, neither prostaglandin E1 nor agonist was added to the PRP. RP was mixed with the tick salivary gland extract sample for 15 min and then added to the coated wells. After a 45-min incubation period at room temperature, the PRP was aspirated from the wells, and the wells were washed twice with adhesion solution (40 mM Tris-HCl, 150 mM NaCl, 0.5% bovine serum albumin, 0.1% glucose, 4 mM MgCl2, and 2 mM CaCl2) and two times with TBS containing 0.1% CaCl2 and MgCl2 (40 mM Tris-HCl, 150 mM NaCl, 4 mM MgCl2, and 2 mM CaCl2). Adherent platelets were fixed with 10% trichloroacetic acid at 40°C for 40 min and then stained with 1% bromphenol blue in 1% acetic acid for 20 min. Subsequently, the plate was washed four times with 1% acetic acid and air-dried. The bound dye was solubilized with 10 μl Tris, pH 9.2, and then absorbance at 595 nm was measured.

Osteosarcoma—Cells of Adhesion—Cells of osteosarcoma cell line Saos-2 (American Type Culture Collection HTB-85) were cultured in medium A (RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, and 15 mg/liter gentamicin). Cells at ~60% confluence were trypsinized with 0.4 ml of a trypsin solution (2.5 g/liter of Dubesco's phosphate-buffered saline without CaCl2) for 4 min at 37°C, neutralized with medium A, and transferred to a centrifuge tube, and the cells were removed from suspension by gentle centrifugation for 5 min. The cells were suspended in medium A and incubated at 37°C for 30 min to recover from trypsinization. After recovery, cells were washed and suspended in serum-free medium B (RPMI 1640 supplemented with 2 mM L-glutamine and 15 mg/liter gentamicin). Cell density was adjusted to 2 × 107/ml. The cells were incubated with a sample of the tick salivary gland extract at 37°C for 30 min. The cell suspension containing the tick sample was transferred to wells (100 μl/well) of a Dynatech Immulon plate that had been coated overnight at 4°C with 100 μg/ml of vonwetricin (1 μg/ml). After 2 h of adhesion at 37°C, wells were washed three times with Dubesco's phosphate-buffered saline containing CaCl2 (from Sigma). Adherent cells were fixed with 100% methanol for 45 min, then washed three times with Dubesco's phosphate-buffered saline containing CaCl2, and stained with 0.1% bromphenol blue in 1% acetic acid for 1 h. After three washes with 1% acetic acid and air drying, Tris-HCl buffer, pH 9.2, was added (100 μl/well). Absorbance was measured at 595 nm using an enzyme-linked immunosorbent assay reader.

Purification of GPIb-IIa—CPIb-IIa was purified from tick salivary glands of the hard tick D. variabilis by a combination of gel filtration and reverse-phase HPLC. Crude salivary gland extract was prepared as described under “Experimental Procedures.” The extract was applied to a BioGel P-10 gel filtration column and washed with 50 mM ammonium acetate, pH 7.0. Gel filtration fractions 11, 12, and 13 (Fig. 1) contained the most potent inhibitory activity of ADP-induced platelet aggre-
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Antagonism of GPIIb-IIIa—Platelet activity of purified variabilin was tested in platelet aggregation and adhesion assays. Purified variabilin potently inhibited platelet aggregation induced by ADP, with an IC50 of 157 nM (Fig. 5). A high concentration (514 nM) of variabilin completely blocked platelet aggregation. Variabilin also inhibited platelet aggregation induced by collagen and by the thrombin receptor peptide SFFLR-RNP (data not shown). However, variabilin did not inhibit the secretion of dense body contents by washed platelets stimulated with the thrombin receptor peptide SFFLRNP (data not shown).

The adhesion studies were done using PRP. PRP was prepared from blood drawn into o-phenyl-alanyl-prolyl-arginine chloromethyl ketone, a thrombin inhibitor. RPs in plasma were prepared by adding prostaglandin E1 (1 \( \mu \)g/ml) to the PRP. The PRP that was not treated with prostaglandin E1 or agonist contained NPs. Both RPs and NPs adhered to Fg immobilized on microtiter plate wells. The extent of adhesion of RPs was about one-half that of NPs (data not shown). Purified variabilin inhibited the adhesion of platelets to immobilized Fg, with IC50s of 143 and 176 nM for RP and NP, respectively (Fig. 6).

Variabilin also potently inhibited binding of purified human GPIIb-IIIa to immobilized Fg (Fig. 7), with an IC50 of 9 nM. The receptor specificity of variabilin was tested using an osteosarcoma cell adhesion assay. Osteosarcoma cells adhere to Vn via the integrin \( \alpha_v\beta_3 \), which shares a common \( \beta \) subunit with GPIIb-IIIa (integrin \( \alpha_{IIb}\beta_3 \)). Also, \( \alpha_v \) has extensive homology with \( \alpha_{IIb} \) (29, 30). Interestingly, \( \alpha_v\beta_3 \) has been shown to be a receptor for all of the ligands known to bind to \( \alpha_{IIb}\beta_3 \), including Fg, fibronectin, Vn, von Willebrand factor, and most disintegrins tested (31). In our adhesion assay, purified variabilin potently inhibited osteosarcoma cell adhesion to immobilized Vn; the IC50 was 87 nM (Fig. 8). This indicates that variabilin, like most disintegrins, is not specific for GPIIb-IIIa but is also antagonistic to the Vn receptor \( \alpha_v\beta_3 \).

**DISCUSSION**

Platelet aggregation studied in an aggregometer can be induced by a variety of agonists through different pathways, but the final common step of these pathways is the binding of Fg to its receptor GPIIb-IIIa on the platelet surface. Accordingly, the saliva or venom of a variety of animals contains antihemostatic agents that inhibit this process. For example, the leech saliva proteins decorin (20) and ornatins (21) and the snake venom disintegrins (24) are antihemostatic agents that function as...
agonists of GPIIb-IIIa. They compete with Fg for binding to 
PPIIb-IIIa and effectively inhibit platelet aggregation elicited 
by a variety of agonists including ADP, collagen, or thrombin 
(25). Decorsin, ornatin, and most disintegrins have an RGD 
sequence, the well known receptor recognition site present on 
many ligands of integrin receptors, including GPIIb-IIIa.

Ticks are obligate ectoparasites that feed solely on the blood 
of mammals, birds, reptiles, and amphibians. Ticks are classi-
fied into two major families, the Argasidae (soft ticks) and the 
Ixodidae (hard ticks) (32). Ticks utilize a battery of antihemo-
static agents to facilitate the feeding process. Specifically, the 
following antihemostatic agents have been isolated from the 
soft tick *Ornithodoros moubata*: tick anticoagulant peptide 
(33), an inhibitor of coagulation factor Xa; moubatin (34), an 
inhibitor of collagen-induced platelet aggregation; and disagre-
gin, an antagonist of GPIIb-IIIa. Unlike decorsin, ornatin, and 
most of the disintegrins, disagregin does not contain an RGD 
sequence, the amino acid sequence used by the other agents to 
inhibit platelet aggregation. The tick *D. variabilis*, from which 
variabilin was isolated, belongs to the Ixodidae. In contrast to 
disagregin, variabilin does contain an RGD sequence.

In this study, a potent anti-platelet agent, variabilin, was 
purified and characterized extensively. Variabilin is the first 
RGD-containing antagonist of GPIIb-IIIa found in ticks. Vari-
abilin was purified to homogeneity from salivary glands of the 
hard tick D. variabilis by gel filtration and reverse-phase 
HPLC chromatography. The purified variabilin potently inhibi-
imited platelet aggregation induced by the platelet agonists ADP, collagen, and thrombin receptor peptide SFLLRNPe. It also blocked platelet adhesion to immobilized Fg. In addition, it inhibited binding of purified human GPIIb-IIIa to immobilized Fg. The spectrum of action of variabilin was not limited to GPIIb-IIIa since variabilin also interfered with the interaction between Vn and its receptor αvβ3, as indicated by its inhibition of osteosarcoma cell adhesion to immobilized Vn. Together, these results indicate that variabilin is an antagonist of both GPIIb-IIIa and αvβ3, and is a potent inhibitor of platelet aggregation. Thus, variabilin resembles decorin, ornatin, and the disintegrins functionally and mechanistically.

Despite this common mode of action of variabilin, it shares little overall structural homology with other proteins of known sequence. Although the RGD sequence is conserved in variabilin, its primary sequence has little other homology with decorin, ornatin, and the snake disintegrins. However, variabilin, decorin, ornatin, and the disintegrins all have a high content of cysteine. For example, cysteine accounts for about 11% of the residues of variabilin. This percentage is comparable to cysteine content in decorin (15%), ornatin (12%), and the disintegrins (16–17%). But in contrast to these other proteins that have an even number of cysteine residues, variabilin has an odd number (five) of cysteine residues. Therefore, unlike these other cysteine-rich proteins, not all of the cysteine residues of variabilin are found in disulfide linkage. Despite the presence of an odd number of cysteine residues, variabilin is not found as a dimer.

The utilization of cysteine residues in variabilin is essentially different than it is in the other RGD containing anti-platelet proteins discussed above. In decorin, ornatin, and the disintegrins, each RGD sequence is positioned within a domain with cysteine boundaries. The two cysteine residues that constitute the boundaries of the RGD-containing domain form disulfide bonds with other cysteine residues in the molecule so that a loop containing the RGD sequence is formed. Structural studies revealed that as a consequence of this arrangement, the RGD sequence lies at the apex of the loop (35–37). It has been suggested that this loop presents the RGD sequence to the RGD binding site of GPIIb-IIIa (35). In contrast to this arrangement, the RGD sequence in variabilin is not bracketed by cysteine residues. Although the RGD sequence of variabilin is preceded by a cysteine three residues upstream (not four residues upstream as in the other proteins), there is no cysteine residue downstream of its RGD sequence. Thus, the RGD sequence of variabilin is not positioned in a domain with cysteine boundaries as occurs in decorin, ornatin, and snake disintegrins. Therefore, the way variabilin presents its RGD sequence to GPIIb-IIIa is probably different than the manner in which it is presented by the leech and snake venom antagonists of GPIIb-IIIa.

In this regard, the RGD sequence in variabilin is bracketed by proline residues, e.g. PRGDP. In decorin, the RGD sequence is also flanked by proline residues, but it has two, rather than one, other residues preceding the downstream proline, e.g. PRGDADP (35). Among the more than 20 snake disintegrins sequenced thus far, at least two disintegrins (kistrin and mambin) have an RGD sequence flanked by proline residues, e.g. PRGDMP (24, 38). It is known that proline residues within a peptide backbone have limited flexibility compared with other residues. Further work is required to reveal if flanking of the RGD sequence by proline residues plays a major role in presenting the RGD sequence of variabilin in an appropriate conformation to GPIIb-IIIa.

It is interesting that variabilin is an effective inhibitor of both GPIIb-IIIa and αvβ3, whereas barbourin (26) and disagregin (25) are apparently specific for GPIIb-IIIa relative to αvβ3. The feeding behavior of hard ticks may provide a rationale for the presence of a broad spectrum anti-integrin inhibitor in their salivary glands. Hard ticks typically feed for a prolonged period, from 7 to 12 days. Therefore, it may be that the hard ticks have a requirement to inhibit the interaction of other cell types with extracellular matrix components during the prolonged feeding period as part of the mechanism by which the feeding tick keeps the blood flowing through its proboscis. In other words, the host may mount a type of wound healing response to mend the damage inflicted by the proboscis of the feeding tick and this nonplatelet, cellular response presumably could be blocked, at least in part, by the ability of variabilin to inhibit the function of αvβ3 and possibly other integrins. Also, there is no obvious advantage for the hard tick to use a GPIIb-IIIa-specific inhibitor of platelet function since the agent is delivered locally in an amount obviously too small to cause a systemic antithrombotic effect to the host. This is apparent since systemic hemorrhaging is not apparent, even in animals supporting the feeding of 300 ticks. Thus, it seems unlikely that other anti-integrin activity of variabilin would seriously damage the host.

In contrast to hard ticks that feed for days, soft ticks feed for a relatively short period of time, about 15 min. This being the case, there may be no advantage for the soft ticks to have a broad action anti-integrin as an anti-platelet agent. Nonetheless, it is not clear why the soft ticks should have a GPIIb-IIIa-specific anti-platelet agent since there appears to be no apparent physiological significance resulting from the inability of disagregin to inhibit the function of αvβ3. Further research is required to reveal whether the occurrence of an apparently GPIIb-IIIa-specific anti-platelet agent in the soft ticks resulted from an evolutionary accident rather than selection.

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