Barbourin

A GPIIb-IIIa-SPECIFIC INTEGRIN ANTAGONIST FROM THE VENOM OF SISTRURUS M. BARBOURI*

(Received for publication, November 8, 1990)

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Sixty-two snake venoms were screened to identify those which specifically inhibit a GPIIb-IIIa receptor-mediated platelet aggregation. Although 52 of these venoms inhibited GPIIb-IIIa, only one of these, from the southeastern pigmy rattlesnake, *Sistrurus m. barbouri, was specific for GPIIb-IIIa and is dependent upon the binding of adhesive proteins to GPIIb-IIIa. This peptide, termed barbourin, was sequenced and found to be highly homologous to other peptides of the viper venom GPIIb-IIIa antagonist family but was the first member which did not contain the Arg-Gly-Asp (RGD) amino acid sequence, believed to be required for inhibition of receptor function. Instead, barbourin contains the sequence, Lys-Gly-Asp (KGD). The conservative Lys for Arg substitution appears to be the sole structural feature which imparts integrin specificity to barbourin, since venom peptide analogs with Lys substitutions were also specific for GPIIb-IIIa. Thus, barbourin represents a new structural model useful for designing potent and GPIIb-IIIa-specific compounds that may have therapeutic value as platelet aggregation inhibitors (5–12).

Platelet aggregation is a key event in thrombus formation and is dependent upon the binding of adhesive proteins to the glycoprotein (GP) IIB-IIIa complex on the platelet surface (1). Although the expression of GPIIb-IIIa is restricted to platelets and megakaryocytes, it has been shown to be a member of a superfAMILY of related receptors known as the integrins, many of which bind to RGD sequences in adhesive proteins and mediate the adhesive interactions of a variety of cells (2–4). Recently, a wide spectrum of true viper and pit viper venoms were screened to contain polypeptides that specifically inhibit the adhesive protein GPIIb-IIIa antagonist family but was the first member which did not contain the Arg-Gly-Asp (RGD) amino acid sequence which they block the adhesive functions of other RGD-dependent integrins, such as the vitronectin receptor(s) (α5β1 and α5β3) and fibronectin receptor (α5β1) and are thus not GPIIb-IIIa-specific (13, 15). Because of this broad reactivity with other integrins, the therapeutic potential of these agents for specifically inhibiting platelet-dependent thrombus formation in vivo may be limited (16, 17). In this report, we describe a new GPIIb-IIIa-specific antagonist from the venom of the southeastern pigmy rattlesnake *Sistrurus m. barbouri and the structural feature of this natural product which imparts novel integrin specificity.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized snake venoms were purchased from either Sigma or Miami Serpentarium Labs (Salt Lake City, UT). Snake venoms from species of the following viper genera were screened in the integrin receptor assays: *Aghistrodon, Atheris, Bitis, Bothrops, Causus, Cerastes, Crotalus, Echis, Erismophis, Lachesis, Pseudocerastes, Sistrurus, Trimeresurus, and Vipera. Sphadex G-50 was obtained from Pharmacia LKB Biotechnology Inc. ADP utilized in the platelet aggregation studies was obtained from Sigma. Sequencing-grade endoproteases Lys-C, Asp-N, and pyrogulanylaminopeptidase were obtained from Boehringer Mannheim. Ultrafiltrates of venom were prepared employing Amicon C-10 microconcentrators using the manufacturer's instructions with crude venom (1 mg/ml) in water.

Solid-phase Integrin Binding Assays—The binding of peptide antagonists to GPIIb-IIIa, α5β1, and α5β3, was determined using solid-phase microtiter assays as described (18, 19). Briefly, purified integrins were added to microtiter wells of enzyme-linked immunosorbent assay plates and blocked with 35 mg/ml BSA to eliminate nonspecific binding (2 h at 30°C). Biotinylated fibrinogen, von Willebrand factor, vitronectin, or fibronectin were added at a final concentration of 20 nM in 20 mM Tris/saline, 1 mM CaCl2, and 1 mg/ml BSA, with or without competing ligand and allowed to incubate for 3 h at 30°C. Bound adhesive ligand was quantitated by the addition of 0.1 ml of anti-biotin antibody conjugated to alkaline phosphatase (Sigma). Nonspecific binding was measured by determining the binding of biotinylated adhesive ligands to BSA-coated wells and was consistently less than 10% of total. All determinations were made in quadruplicate and each peptide was examined a minimum of three times.

Purification of Barbourin, Tergeminin, and Eristicophin—The purification of the snake venom peptides barbourin, tergeminin, and eristicophin was accomplished using the following modifications of previously described methods (5–12). One gram of each venom was solubilized in 0.5 M acetic acid and applied to a column of G-50 (2.5 × 100 cm) and eluted in 0.5 M acetic acid. Fractions from the column were assayed for the ability to inhibit the ADP-induced aggregation of human platelets in platelet-rich plasma, and active fractions were lyophilized and applied to a C18 reversed-phase column (Vydac 218TP54, 0.45 × 25 cm, 300 Å) to obtain homogeneous peptides.

Radioiodination of Barbourin—Barbourin (50 μg) was radiolabeled using lactoperoxidase-catalyzed iodination (20) and purified on C18
reversed-phase high performance liquid chromatography as described above. The specific activity of this material was $1.35 \times 10^4$ cpn/mol.

**Peptide Synthesis**—[Glu\(^{3}\),Leu\(^{4}\),Cys\(^{8}\)]Barbourin-(28-73) and [Lys\(^{3}\)]eristicophin-(4-51) were prepared by automated peptide synthesis using an Applied Biosystems (Foster City, CA) model 431A peptide synthesizer employing 1-hydroxybenzotriazole-activated butyloxycarbonyl-protected amino acids using the manufacturer's instructions. Double-coupling of all residues followed by capping with acetic anhydride was performed during chain assembly. Cleavage and refolding of the synthetic peptides was performed as described by Garsky et al. (21). The purity of each of the synthetic peptides was established by analytical RPLC on Vydis C\(_2\) columns. Purified synthetic peptides were reduced (6 M guanidine, 30 mM dithiothreitol, pH 7.5, at room temperature) and carboxamidomethylated for amino acid sequence determination (7, 11). Single Edman degradation runs of each of the synthetic peptides were obtained using an Applied Biosystems model 473A protein sequenator employing the manufacturer's instructions and these confirmed the sequences of the analogs (22).

**RESULTS AND DISCUSSION**

Newly developed solid-phase integrin ligand binding assays were used to screen a variety of snake venoms for their ability to block adhesive protein binding to purified integrins (18, 19). Sixty-two commercially available viper venoms from 14 different genera were initially examined. Ultrafiltrates of the crude venoms ($M < 10,000$) were employed in this screen in order to remove proteolytic enzymes which are present in high concentrations in most viper venoms and which have the potential of degrading the integrin proteins or adhesive ligands used in the assays (23). Fifty-two of the venoms assayed contained potent GPIIIb-IIIa antagonist activity, which is consistent with previous reports of the presence of GPIIIb-IIIa inhibitors in many viper venoms (5-12). Of these, 51 also inhibited the binding of vitronectin to $\alpha_{\beta_3}$, an integrin that has the same $\beta$ subunit as GPIIIb-IIIa and is also very sensitive to inhibition by RGD-containing peptides (7, 11). Single Edman degradation runs of each of the synthetic peptides were obtained using an Applied Biosystems model 473A protein sequenator employing the manufacturer's instructions and these confirmed the sequences of the analogs (22).

**TABLE I**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Inhibition of adhesive protein binding to integrins by snake venom peptides and analogs</th>
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<tbody>
<tr>
<td></td>
<td>IC(_{50}) nM</td>
</tr>
<tr>
<td>Barbourin</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Tergeminin</td>
<td>&gt;600</td>
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<tr>
<td>Eristicophin (4-51)</td>
<td>&gt;100</td>
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<tr>
<td>[Lys(^{3})]Eristicophin</td>
<td>&gt;100</td>
</tr>
<tr>
<td>[Glu(^{3}),Leu(^{4}),Cys(^{8})]Barbourin-(28-73)</td>
<td>10</td>
</tr>
<tr>
<td>Echistatin</td>
<td>10</td>
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Inhibition of adhesive protein binding to purified integrins immobilized into 96-well plates in the presence of various concentrations of snake venom peptides and analogs. The concentration of peptide which inhibited receptor binding by 50% (IC\(_{50}\)) was determined from at least three experiments. The IC\(_{50}\) value for platelet aggregation is the concentration necessary to inhibit platelet aggregation in platelet-rich plasma to 50% of control aggregation induced by ADP (20 $\mu$M).

minin from *S. c. tergeminus*, and eristicophin from *E. macmahoni*, were obtained from the individual crude venoms using a combination of gel filtration and reversed-phase liquid chromatography purification protocols. The relative potencies of these purified peptides in inhibiting the binding of fibrinogen to GPIIIb-IIIa and vitronectin to $\alpha_{\beta_3}$ are shown in Fig. 1 and Table I. A similar pattern of GPIIIb-IIIa-selective inhibition was observed with von Willebrand factor, which also binds to GPIIIb-IIIa and $\alpha_{\beta_3}$. Barbourin, tergeminin, and eristicophin weakly inhibited the binding of fibronectin to $\alpha_{\beta_3}$, compared with the disintegrin echistatin (Table I).

Amino acid analysis of purified barbourin, tergeminin, and eristicophin revealed compositions that were very similar to each other and to other members of the snake venom disintegrin family (data not shown). The complete amino acid sequences of these peptides were determined utilizing reduced and carboxamidomethylated peptide fragments generated from digestions employing endoproteases Lys-C and Asp-N and are illustrated for barbourin in Fig. 2A. Barbourin contains 73 amino acids including 12 cysteines. Although barbourin has significant amino acid sequence homology with previously described viper venom disintegrins (Fig. 2B), a striking struc-
Barbourin: A GPIIb-IIIa-specific Disintegrin

The amino acid sequence of 73 residues was determined from the analysis of reduced and carboxamidomethylated peptide and peptide fragments generated employing endoprotease Lys-C and endoprotease Asp-N digestions. Peptide fragments were purified using reversed-phase liquid chromatography and sequenced by automated Edman degradation of peptides using an Applied Biosystems 477 gas-phase sequenator.

The single-letter amino acid code is used. The Z at the NH2 terminus of eristicophin refers to a pyroglutamyl residue determined from pyroglutamylaminopeptidase digestion. Conserved residues are boxed.

**Fig. 2.** A, amino acid sequence of the GPIIb-IIIa antagonist from S. m. barbouri. The amino acid sequence of 73 residues was determined from the analysis of reduced and carboxamidomethylated peptide and peptide fragments generated employing endoprotease Lys-C and endoprotease Asp-N digestions. Peptide fragments were purified using reversed-phase liquid chromatography and sequenced by automated Edman degradation of peptides using an Applied Biosystems 477 gas-phase sequenator.

B, sequence alignment of viper venom GPIIb-IIIa antagonists. Sequences of barbourin, tergeminin, and eristicophin are compared with the previously described sequences of trigramin (5-12), kistrin (5-12), kistrin (9), applaggin (9), and echistatin (7). The single-letter amino acid code is used. The Z at the NH2 terminus of eristicophin refers to a pyroglutamyl residue determined from pyroglutamylaminopeptidase digestion. Conserved residues are boxed.

**Fig. 3.** Binding of 125I-barbourin to purified human GPIIb-IIIa. Labeled 125I-barbourin was incubated with purified GPIIb-IIIa or the vitronectin receptor, αβ3, immobilized on microtiter plates in the presence or absence of EDTA, to determine nonspecific binding. Measurements were made in quadruplicate. Inset, the dissociation constant (Kd) was calculated to be 18 nM as determined by the method of Scatchard (26) using the computer program LIGAND (33).

The amino acid sequence of tergeminin was also determined (Fig. 2B) (7). Tergeminin and eristicophin contain the RGD sequence and are not integrin specific (Fig. 1). Barbourin does not bind to purified GPIIb-IIIa. The Lys for Arg substitution in barbourin strongly suggests that the receptor recognition sequence of this peptide was responsible for its novel selectivity for GPIIIb-IIIa. To demonstrate a direct interaction of barbourin with GPIIb-IIIa, barbourin was radiolabeled using lactoperoxidase-catalyzed iodination and purified by RPLC. 125I-Barbourin was found to bind to purified GPIIb-IIIa in a specific and saturable manner with high affinity. This binding was greater than 80% reversible and was therefore analyzed by the method of Scatchard (26) which revealed a dissociation constant (Kd) of 18 nM (Fig. 3). This is similar to other RGD-containing peptides such as trigramin, kistatin, applaggin, and echistatin (5-12). 125I-Barbourin bound to unstimulated washed human platelets with an apparent dissociation constant (Kd) of 4.0 × 10-7 M and to platelets stimulated with ADP (10 μM) with an apparent Kd = 1.4 × 10-7 M. These values are very similar to those reported by Savage et al. (13) for the binding of echistatin to unactivated and ADP-stimulated platelets. 125I-Barbourin does not bind to purified αβ3 (Fig. 3) or fibrinogen (data not shown). These data indicate that the inhibition of fibrinogen binding was due to barbourin’s ability to bind to GPIIIb-IIIa.

The Lys for Arg substitution in barbourin strongly suggests that the receptor recognition sequence of this peptide was responsible for its novel selectivity for GPIIIb-IIIa. In order to demonstrate this directly, a synthetic truncated form of barbourin, [Glu28,Leu41,Cys61]barbourin-(28-72) and [Lys30]eristicophin-(4-51) were assayed for integrin specificity using the solid-phase microtiter plate assay as described under "Materials and Methods." VN/VNR refers to vitronectin binding to αβ3. Various doses of both peptides were allowed to compete for the binding of fibrinogen to GPIIb-IIIa and vitronectin to αβ3. [Glu28,Leu41,Lys30]barbourin-(28-73); [Lys30]eristicophin (data not shown). These data indicate that the inhibition of fibrinogen binding was due to barbourin’s ability to bind to GPIIb-IIIa.

**Fig. 4.** Selective inhibition of GPIIb-IIIa by KGD-substituted peptides. The truncated barbourin peptide, [Glu28,Leu41,Cys61]barbourin-(28-72) and [Lys30]eristicophin-(4-51) were assayed for integrin specificity using the solid-phase microtiter plate assay as described under "Materials and Methods." VN/VNR refers to vitronectin binding to αβ3. Various doses of both peptides were allowed to compete for the binding of fibrinogen to GPIIb-IIIa and vitronectin to αβ3. [Glu28,Leu41,Lys30]barbourin-(28-73); [Lys30]eristicophin (data not shown). These data indicate that the inhibition of fibrinogen binding was due to barbourin’s ability to bind to GPIIb-IIIa.

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Barbourin: A GPIIb-IIIa-specific Disintegrin

...prevents favorable interactions of these peptides with primary amine function on Lys could also result in unfavorable charge interactions within the $\alpha_\beta_3$ binding pocket.

Earlier studies have shown that peptides containing the KGDX sequence are much poorer antagonists of integrin function than RGD-containing peptides (5, 27–29). As reported previously we also find that linear KGDX peptides are approximately 5-fold less active as antagonists of GPIIb-IIIa than the corresponding linear RGDX peptides (not shown).

Thus, it has been widely accepted that the Arg of the RGDX sequence is an important requirement for high affinity binding to GPIIb-IIIa. These observations also suggest that specific binding of ligands to GPIIb-IIIa may occur through the medium instead of the high affinity binding pocket. These two receptors have not been previously implicated as GPIIb-IIIa recognition sequences.

Barbourin is also a weak inhibitor of the interaction of fibronectin with $\alpha_\beta_5$, but in this case is similar to tergeminin and eristicophin, which are very different from barbourin. It is likely that the Trp residue at the $\alpha$ position prevents favorable interactions of these peptides with $\alpha_\beta_5$.

The structural basis of the lack of interaction of barbourin with $\alpha_\beta_5$ is unclear, particularly since both GPIIb-IIIa and $\alpha_\beta_5$ share the $\beta$ subunit and the binding of at least four RGD-containing adhesive proteins is possible. It is possible that the combination of different $\alpha$ subunits with $\beta$ leads to slightly altered ligand binding pockets in these two receptors. These alternative structures could dictate different charge or size requirements for the binding of ligands and may be the reason why Lys-substituted peptides do not bind to $\alpha_\beta_5$. Specifically, the extra methylene group of the Lys side chain may result in increased steric hinderance, and the decreased $pK_a$ of the primary amine function on Lys could also result in unfavorable charge interactions within the $\alpha_\beta_3$ binding pocket.

It has been demonstrated previously that the binding of RGD-containing peptides to fibronectin and vitronectin receptors can be altered by influencing the conformational display of the Arg-Gly-Asp-X sequence (31). However, barbourin is the first example of a highly selective integrin ligand that owes its unique specificity to simple side chain alteration of the RGD recognition signal. Barbourin may thus serve as an important model for designing highly potent and selective GPIIb-IIIa antagonists that can be used as antiplatelet agents (16, 32).

REFERENCES