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Specific inhibition of binding of antistasin and [A^{103,106,108}] antistasin 93–119 to sulfatide (Gal(3-SO4)β1-1Cer) by glycosaminoglycans

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Leech-derived antistasin is a potent anticoagulant and antimetastatic protein that binds sulfatide (Gal(3-SO4)β1-1Cer) and sulfated polysaccharides. In this study, the synthetic fragment [A^{103,106,108}] antistasin 93–119, which corresponds to the carboxyl terminus, showed specific and saturable binding to sulfatide. Binding was competitively blocked by glycosaminoglycans (GAGs) in the order: dextran sulfate 5000 ≈ dextran sulfate 500 000 > heparin> dermatan sulfate ≫ chondroitin sulfates A and C. This rank order of inhibitory potency was identical to that observed with whole antistasin. We suggest that residues 93–119 of antistasin represent a critical domain for binding GAGs and sulfated glycolipids.

Antistasin; Ghilanten; *Haementeria*; Sulfatide; Glycosaminoglycans; Leech

1. INTRODUCTION

Antistasin is a 119-amino acid leech protein from *Haementeria officinalis* [1]. This protein, and a similar one from *Haementeria ghilianii* [2], inhibit the blood clotting enzyme Factor Xa. These inhibitors contain a 2-fold internal repeated homology [3,4]. The amino-terminal domain contains the inhibitory residues that block the catalytic site of Factor Xa [3,4]. The carboxyl-terminal half contains a basic amino acid-rich tail that fits a consensus motif for binding glycosaminoglycans(GAGs) [5]. At present, the domain(s) that mediates the specific interactions of antistasin with GAGs and sulfated glycolipids remains to be determined. In this study,we demonstrate that [A^{103,106,108}] antistasin 93–119 mimics the interaction of whole antistasin with sulfatide and sulfated polysaccharides.

2. EXPERIMENTAL

[A^{103,106,108}] antistasin 93–119 was synthesized as recently described [6] in which alanine for cysteine substitutions were made at positions 103, 106 and 108 in order to prevent covalent aggregation during purification. Peptides purity was established by analytical high performance liquid chromatography, quantitative amino acid analysis [7] and fast atom bombardment mass spectrometry. Antistasin was purified from fresh salivary glands from *Haementeria officinalis* as described in [1] and purity was established as described in [2]. Antistasin and [A^{103,106,108}] antistasin 93–119 (50 μg) were radioiodinated with Na[^{125}I] (17 Ci/mmol, Amersham-Searle, Arlington Heights, IL) using the chloramine-T method [8]. The reaction mixture was desalted on a Bio Gel P-2 column equilibrated in phosphate buffered saline, pH 7.4 (PBS); 0.5 ml fractions were collected in teflon coated tubes containing 100 μl of PBS and 6 mg/ml BSA. The labeled samples were stored at −80°C and a fresh tube thawed for each experiment.

The wells of Immulon 4 Removawell polystyrene microtitre plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 200 μl of 100 μg/ml Gal(3-SO4)β1-Cer (sulfatide; Sigma, St. Louis, MO) in methanol. The solvent was evaporated with nitrogen and the plate subjected to high vacuum for 20–30 min. Wells were blocked for 2 h with 250 μl of 1% bovine serum albumin (BSA) in standard buffer (10 mM HEPES, 0.15 M NaCl, pH 7.4), aspirated and washed 4-times. Next, increasing amounts of ^{125}I-labeled antistasin or ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 were added. After 3–4 h at room temperature the wells were aspirated and washed as above. ^{125}I-labeled antistasin and ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 bound to sulfatide were then determined by gamma counting. For competition studies, increasing amounts of GAGs were added, followed by a constant amount of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 or ^{125}I-labeled antistasin. The wells were incubated and washed as described above until the counts in the wash were near to background. The amount of peptide bound to sulfatide was determined by gamma counting.

Heparin binding was assessed by adding increasing amounts of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 to Eppendorf tubes containing 100 μl of heparin agarose (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, MD) in a final volume of 250 μl of 50 mM HEPES, 0.1 M NaCl, pH 7.4. The tubes were incubated for 5 min at room temperature, centrifuged and counts in the supernatant were determined.

3. RESULTS AND DISCUSSION

Fig. 1 shows that the binding of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 to Gal(3-SO4)β1-1Cer was saturable and specific as negligible binding occurred in the absence of sulfatide. As shown, this peptide contains

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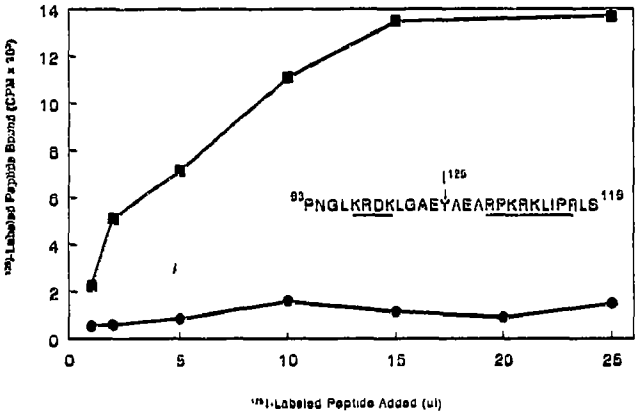

Fig. 1. The binding of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 to sulfatide. Microtiter wells were coated (■) or not coated (●) with sulfatide.

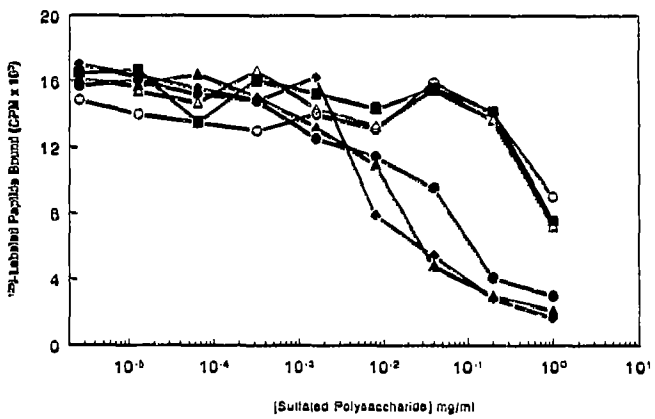

Fig. 3. The effect of various glycosaminoglycans on the binding of [A^{103,106,108}] antistasin 93–119 to Gal(3-SO4)β1-Cer. Dextran sulfate 5000 (♦); dextran sulfate 500 000 (▲); heparin (●); dermatan sulfate (△); chondroitin sulfates A (■) and C (○).

2 basic-rich amino acid regions (underlined) that potentially mediate antistasin's interaction with sulfated glycolipids and GAGs. Radioiodination occurred at Tyr-105, 3 and 4 residues removed from these domains.

Fig. 2 shows that the binding of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 to heparin agarose was specific for the GAG as unlabeled heparin caused its release into the supernatant. Thus, Figs. 1 and 2 demonstrate that [A^{103,106,108}] antistasin 93–119 binds both to sulfatide and heparin. As GAGs are known to block the antistasin–sulfatide interaction [9] the relative ability of various sulfated polysaccharides to block the binding of [A^{103,106,108}] antistasin 93–119 to Gal(3-SO4)β1-1Cer was examined. The results of a representative experiment are shown in Fig. 3 and the data from 4 such experiments are summarized in Table I. The relative order of the inhibitory potencies (dextran sulfate 5000 = dextran sulfate 500000 > heparin > dermatan sulfate ≫ chondroitin sulfates A and C) and their ratios (dextran sulfate: heparin and dextran sulfate: dermatan sulfate) were nearly identical to those reported to block the binding of antistasin to sulfatide [9] and ^{125}I-labeled heparin binding to [A^{103,106,108}] antistasin 93–119 [6]. Thus antistasin and its carboxyl-terminal tail (residues 93–119) show the same quantitative relationships between their GAG and sulfatide binding properties. The relative abilities of dextran sulfate, heparin and dermatan sulfate to suppress the binding of antistasin to sulfatide are shown in Fig. 4. Dextran sulfate and heparin were considerably more potent than dermatan sulfate, whilst chondroitin sulfates A and C (not shown) were inactive at all concentrations tested.

In summary, this study demonstrates that residues 93–119 of antistasin mimics the interaction of antistasin with sulfatide and sulfated polysaccharides. Site-directed mutational studies should clarify further the functional role of this domain in the in vivo anticoag-

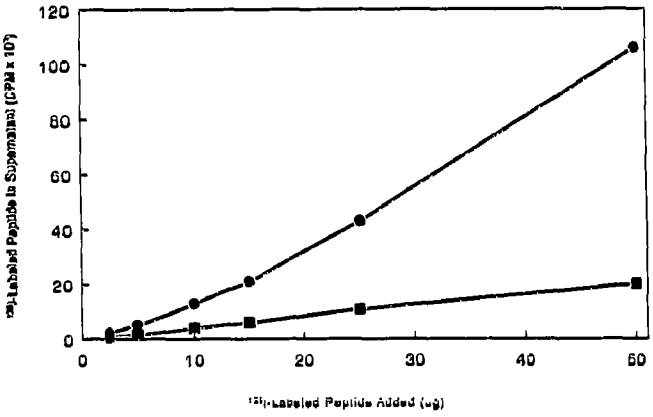

Fig. 2. The binding of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 to heparin agarose in the absence (■) and presence (●) of 10 mg/ml heparin.

Table I

Effect of sulfated polysaccharides on the binding of ^{125}I-labeled [A^{103,106,108}] antistasin 93–119 to sulfatide

GAG	IC_{50} (μg/ml)[a]		
	Peptide*/ sulfatide[b]		Heparin**/ peptide[c]
Heparin	90	(16)	(100)
Dextran sulfate 5000	8	(1.4)	(7)
Dextran sulfate 500 000	9	(1.5)	(8)
Dermatan sulfate	950	(195)	(1000)
Chondroitin sulfate A	>1000	(>500)	(>1000)
Chondroitin sulfate C	>1000	(>500)	(>1000)

[a] Concentration of GAG giving 50% inhibition of binding.
[b] Values in parentheses reported by Holt et al. [9] for whole antistasin binding to sulfatide.
[c] Data in parentheses are those of Manley et al. [6].
* Indicates radioiodinated [A^{103,106,108}] antistasin 93–119 bound to Gal(3-SO4)β1-1Cer that is displaced by the various GAGs.
** Indicates radioiodinated heparin bound to [A^{103,106,108}] antistasin 93–119 that is displaced by the various GAGs.

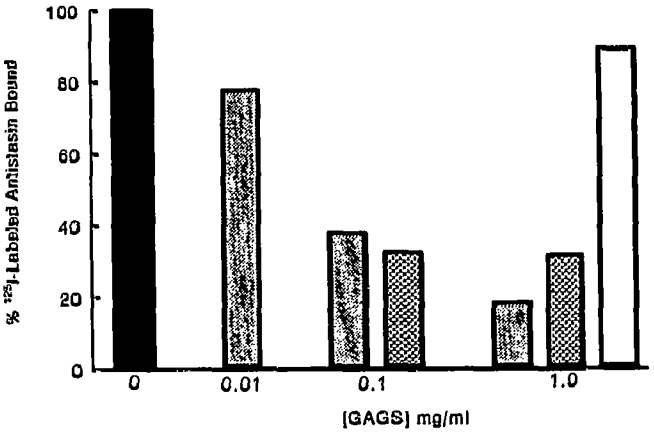

Fig. 4. The effect of various glycosaminoglycans on the binding of ^{125}I-labeled whole antistasin to sulfatide. Solid bar, control (no competitor added); striped bar, dextran sulfate 5000; cross-hatched bar, heparin; open bar, dermatan sulfate. No inhibition was observed with chondroitin sulfates A and C at 1 mg/ml.

ulant, antimetastatic and pharmacokinetic behaviour of the inhibitor.

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