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DEMONSTRATION THAT [A^{103,106,108}] ANTISTASIN 93-119 INHIBITS THE SPECIFIC BINDING OF ANTISTASIN TO SULFATIDE [Gal(3-SO4)β1-1Cer]

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Antistasin is a 119 amino acid heparin-binding protein from the leech Haementaria officinalis which has anticoagulant and antimetastatic properties. A series of peptides representing the basic amino acid-rich domains of the amino- and carboxyl-terminal regions of the inhibitor were synthesized by solid-phase peptide chemistry and their ability to bind sulfated glycolipids was investigated. The findings show that [A^{103,106,108}] antistasin 93-119 has high affinity for sulfatide and inhibits the specific interaction of whole antistasin with [Gal(3-SO4)β1-1Cer]. We conclude that the 93-119 region is a critical domain that mediates the interaction of antistasin with sulfated glycolipids.

Antistasin (1), and the sequence-related protein ghilanten (2), are potent anticoagulant-antimetastatic salivary gland proteins of the proboscis leeches Haementaria officinalis and Haementaria ghilianii, respectively. These proteins block the active site of Factor Xa, a critical enzyme involved in blood coagulation (1,2). These inhibitors exhibit a high affinity for heparin. The carboxyl-terminal residues 93-119 have a sequence organization of basic amino acids similar to those which comprise the heparin-binding domains of apolipoproteins B, E, and antithrombin III (3,4). Furthermore, it was proposed that residues 32-48 and 87-101 were important for binding to heparin and to sulfated glycolipids based on their limited homology with sequences in the sulfatide-binding proteins properdin and thrombospondin (5,6). To investigate the contributions of each of these regions to the sulfated glycoconjugate-binding properties of antistasin, the respective peptides were synthesized and evaluated for their ability to interact specifically with sulfatide. These studies show that the carboxyl-terminal fragment [A^{103,106,108}] antistasin 93-119 selectively blocks the antistasin-sulfatide interaction.

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METHODS AND MATERIALS

Peptide synthesis was accomplished on a 0.5 mmol scale by solid-phase methods using an Applied Biosystems model 430A peptide synthesizer and the appropriate Nα-t-Boc-amino acid Pam resin (Applied Biosystems, Foster City, CA). All Nα-t-Boc protected amino acids were double coupled first as their symmetrical anhydrides in dimethylformamide and then in dichloromethane except for arginine, asparagine, and glutamine which were double coupled using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The side chain protection of the amino acids was as follows: Arg (Tos), Lys (2-ClZ), Glu (Bzl), Tyr (2-Br-Z), Ser (Bzl), Thr (Bzl), Asp (Chx), Cys (pMeBzl), His (Tos). The peptides were cleaved from the resin and deprotected in anhydrous hydrogen fluoride (HF) (containing 5% anisole) at -5°C for 30 min. After removal of HF in vacuo, the peptides were extracted from the resin with 30% aqueous acetic acid and 25% aqueous acetonitrile. Cyclization of peptides 37-48 and 92-103 was accomplished by oxidation of the Cys side chains with $K_3Fe(CN)_6$. The peptides were purified by preparative HPLC on a Dynamax C_{18} column (21.4 x 250 mm, Rainin Instruments, Woburn, MA) using various gradients of acetonitrile in 0.1% aqueous trifluoroacetic acid. The purity and identity of the peptides were 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 of the leech Haementaria officinalis according to methods described previously (1). Purity was confirmed by SDS polyacrylamide gel electrophoresis, amino acid analysis, verification of its blocked amino terminus by automated gas-phase microsequence analysis and by determination of its specific anti-Factor Xa activity as described in (2).

Binding studies were performed with Immulon 4 Removawell polystyrene microtitre plates (Dynatech Laboratories, Inc., Chantilly, VA). Wells were coated with 200 µl of 100 µg/ml solution of [Gal(3-SO4)β1-1Cer] (sulfatide, Sigma, St. Louis, MO.) in methanol, the solvent evaporated with nitrogen gas and then lyophilized for 20-30 min. Wells were blocked with 250 ul of 1% bovine serum albumin (BSA) solution in standard buffer (10 mM HEPES, 0.15 M NaCl pH 7.4) for 2 hours, aspirated and then washed 4 times with 250 ul of standard buffer. To demonstrate saturable and specific binding to sulfatide, increasing amounts of ^{125}I-labeled antistasin or ^{125}I-labeled [$A^{103,106,108}$] antistasin 93-119 were added to the wells. After 3-4 hours at room temperature the wells were aspirated and washed to remove unbound peptides. The amount of ^{125}I-labeled antistasin or ^{125}I-labeled [$A^{103,106,108}$] antistasin 93-119 bound to the wells was determined by gamma counting. For competition studies, increasing concentrations of unlabeled peptides were added followed by the addition of a constant amount of either ^{125}I-labeled [$A^{103,106,108}$] antistasin 93-119 or ^{125}I-labeled antistasin. The wells were incubated for 3-4 h, washed and aspirated 4-8 times with 250 µl standard buffer as appropriate to reduce counts in the wash to background. The wells were then detached from the plate and subjected to gamma counting to determine the degree of binding inhibition by each peptide.

Antistasin and [$A^{103,106,108}$] antistasin 93-119 were radioiodinated with Na ^{125}I (17 Ci/mmol, Amersham-Searle, Arlington Heights, IL) using a modification of the chloramine-T method (8). Na ^{125}I (0.5 mCi) was added to 50 µg of antistasin or peptide in 0.5 M sodium phosphate pH 7.5. The reaction was started by the addition of 10 µl of chloramine-T (1.25 mg/ml in 0.5 M phosphate buffer) and after 30 s at 24°C it was stopped by adding 25 µl of sodium metabisulfate (1.25 mg/ml in 0.5 M phosphate buffer). The 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 then stored at -80°C and a fresh tube thawed for each experiment.

RESULTS

The purpose of these studies was to determine the region(s) in antistasin that binds to sulfated glycoconjugates. Peptides of the amino- and carboxyl-

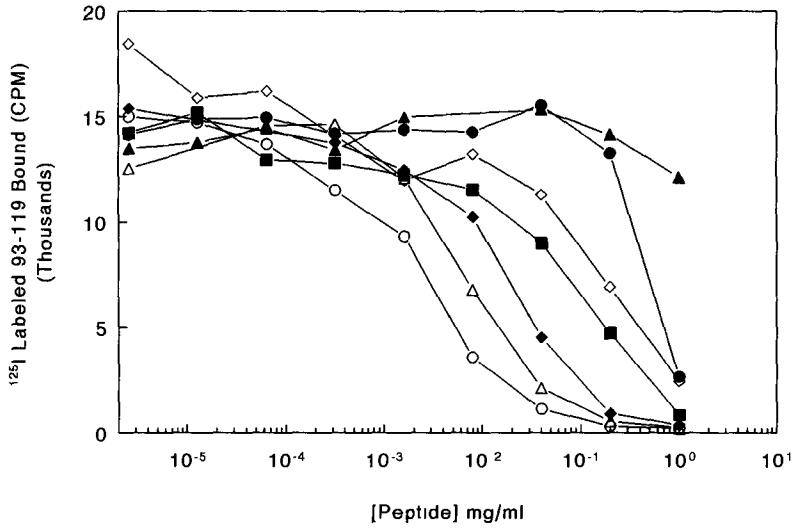

Figure 1. Ability of various antistasin peptides to inhibit the binding of ^{125}I-labeled [$A^{103,106,108}$] antistasin 93-119 to sulfatide. (○), [$A^{103,106,108}$] antistasin 96-119; (△), [$A^{103,106,108}$] antistasin 93-119; (◆), antistasin 109-119; (■), cyclic antistasin 37-48; (◇), [$A^{45-65,88,92}$] antistasin 87-101; (●), cyclic antistasin 92-103; (▲), acetyl hirudin 45-65.

terminal regions of the inhibitor were synthesized and, where indicated, alanine for cysteine substitutions were made to prevent covalent aggregation. The peptides were then examined for their abilities to compete with either ^{125}I-labeled [$A^{103,106,108}$] antistasin 93-119 or with ^{125}I-labeled whole antistasin for the sulfatide [Gal(3-SO4)β1-1Cer]. [$A^{103,106,108}$] antistasin 93-119 was chosen for the initial study as this fragment binds heparin and contains a single tyrosine for radioiodination that is 3-4 residues removed from the clusters of basic residues that mediate its binding to negatively charged molecules (3). Fig. 1 is a representative experiment showing the dose-dependent inhibition of binding of ^{125}I-labeled [$A^{103,106,108}$] antistasin 93-119 to sulfatide by various unlabeled antistasin peptides. As a control, the negatively charged, carboxyl-terminal fragment of hirudin (9), acetyl hirudin 45-65 (Ac-T-P-K-P-Q-S-H-N-D-G-D-F-E-E-I-P-E-E-Y-L-Q), was included and showed no ability to compete with sulfatide for the labeled antistasin peptide.

The amino acid sequences of the various peptides and their sulfatide binding activities are summarized in Table 1. The inhibitory potencies (IC_{50}'s) of [$A^{92,103,106,108}$] antistasin 87-119, [$A^{103,106,108}$] antistasin 93-119, [$A^{103,106,108}$] antistasin 96-119 and antistasin 109-119 were 0.1, 0.9, 0.9 and 13 μM, respectively. [A^{92}] antistasin 87-101 showed limited activity (IC_{50} = 150 μM) indicating that [A^{92}] antistasin 87-101 and antistasin 109-119 (IC_{50} = 13 μM) contribute nonadditively to enhance the sulfatide-binding affinity of [$A^{92,103,106,108}$] antistasin 87-119 (IC_{50} = 0.1 μM). Thus, the residues

TABLE 1. STRUCTURE AND SULFATIDE BINDING ACTIVITY OF LEECH PEPTIDES

Carboxy-Terminal Peptides	IC_{50} (μM)
^{87}N-A-R-K-T-A-P-N-G-L-K-R-D-K-L-G-A-E-Y-A-E-A-R-P-K-R-K-L-I-P-R-L-S^{119}	0.1
^{87}N-A-R-K-T-A-P-N-G-L-K-R-D-K-L^{101}	150
^{92}C-P-N-G-L-K-R-D-K-L-G-C^{103} (cyclic)	410
^{93}P-N-G-L-K-R-D-K-L-G-A-E-Y-A-E-A-R-P-K-R-K-L-I-P-R-L-S^{119}	0.9
^{96}L-K-R-D-K-L-G-A-E-Y-A-E-A-R-P-K-R-K-L-I-P-R-L-S^{119}	0.9
^{109}R-P-K-R-K-L-I-P-R-L-S^{119}	13
Amino Terminal Peptides	
^{32}R-A-R-V-H-A-P-H-G-F-Q-R-S-R-Y^{46}	2.0
^{37}C-P-H-G-F-Q-R-S-R-Y-G-C^{48} (cyclic)	53

between 109-119 contribute to the high-affinity domain of the 87-119 region. [$A^{33,37}$] antistasin 32-46 had an IC_{50} = 2 μM suggesting its possible importance whereas cyclic antistasin 37-48 showed marginal activity (IC_{50} = 53 μM). Cyclization around the basic regions of antistasin 37-48 and antistasin 92-103 (IC_{50} = 410 μM) by disulfide bond formation did not enhance activity.

It was reasoned that if the carboxyl-terminal region of antistasin contributes to the sulfatide binding activity of the inhibitor then synthetic fragments of this domain should compete with whole antistasin in the assay. Figure 2 shows that 0.01 mg/ml of [$A^{88,92,103,106,108}$] antistasin 87-119 and [$A^{103,106,108}$] antistasin 93-119 blocked the binding of whole antistasin to sulfatide by 25-35%. At 1.0 mg/ml [$A^{33,37}$] antistasin 32-46 still showed no activity, while [$A^{88,92,103,106,108}$] antistasin 87-119 and [$A^{103,106,108}$] antistasin 93-119 inhibited binding by 50-70%.

DISCUSSION

We previously demonstrated that residues 93-119 of the carboxyl terminus of antistasin bound ^{125}I-labeled heparin with high affinity and with saturable kinetics (3). Binding was enhanced at physiological pH and ionic strength and was blocked by various glycosaminoglycans (GAGS) in the order: dextran sulfate > heparin > dermatan sulfate ≥ chondroitin sulfate A and C (10). As this rank order of inhibitory potency was identical to that for the GAGS reported to block whole antistasin binding to [Gal(3-S04)β1-1Cer] (5) we investigated whether the carboxyl-terminal fragment encoded sufficient structural information to bind to the sulfated glycolipid and also to compete specifically

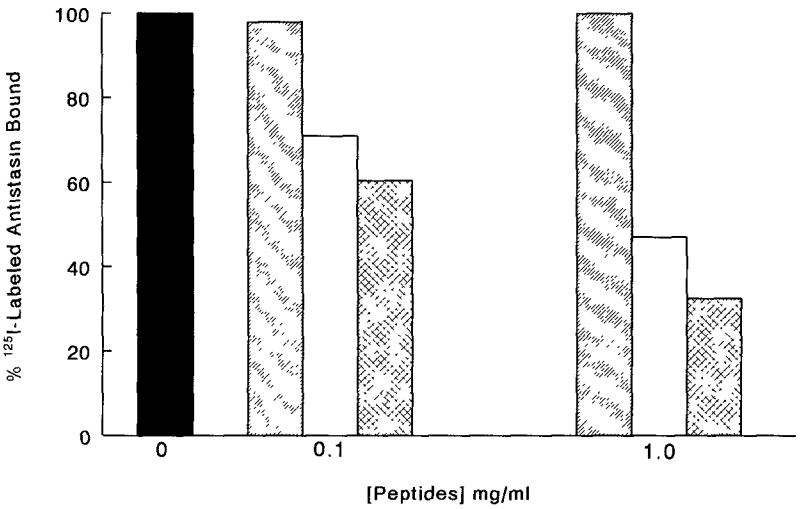

Figure 2. [$A^{92,103,106,108}$] antistasin 87-119 and [$A^{103,106,108}$] antistasin 93-119 inhibit the binding of whole antistasin to sulfatide. The solid bar is 100% binding with no inhibitor added corrected for nonspecific binding in the presence of excess dextran sulfate. The striped bar is with [$A^{33,37}$] antistasin 32-46 added. The open bar is with [$A^{88,92,103,106,108}$] antistasin 87-119 added and the cross-hatched bar is with [$A^{103,106,108}$] antistasin 93-119 added.

with the leech protein for sulfatide in the assay. The present study demonstrates that residues 109-119 contain a high-affinity domain for binding sulfatide and residues 93-119, at 10 μg/ml, contain the necessary recognition elements to compete specifically with whole antistasin for [Gal(3-SO4)β1-1Cer]. Although peptide 32-46 showed a low micromolar affinity for sulfatide, the peptide was unable to inhibit the antistasin-sulfatide interaction at 1 mg/ml. These findings suggest that the carboxyl-terminal residues 93-119 represent a major determinant of the sulfatide binding region(s) of antistasin. Further studies with protein mutants lacking the carboxyl terminus should clarify the role of this domain in sulfatide binding.

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