Chimeric Antithrombin Peptide

CHARACTERIZATION OF AN ARG-GLY-ASP (RGD)- AND HIRUDIN CARBOXYL TERMINUS-CONTAINING SYNTHETIC PEPTIDE

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We investigated the properties of an artificial chimeric peptide that contains an Arg-Gly-Asp (RGD)-tripeptide, the versatile cell recognition signal of extracellular matrix protein components, coupled to a carboxy-terminal fragment of the highly specific α-thrombin inhibitor, hirudin (residues 53–64): WGRGDSDANGDFEEPEEYL (RGD-hirudin53–64). Hirudin53–64 and RGD-hirudin53–64 inhibited the fibrinogen clotting activity of α-thrombin and prolonged the activated partial thromboplastin time of human plasma. In addition, both peptides afforded total protection to thrombin from trypsinolysis. Neither hirudin53–64 nor RGD-hirudin53–64 dramatically interfered with the thrombin-antithrombin inhibition reaction either in the absence or presence of added heparin. α-Thrombin-induced platelet aggregation was effectively inhibited by hirudin53–64 and RGD-hirudin53–64. Unlike hirudin53–64, RGD-hirudin53–64 in solution inhibited integrin-mediated endothelial cell and fibroblast cell attachment to polystyrene wells in the presence of fetal bovine serum. Collectively, our results demonstrate that RGD-hirudin53–64 has anticoagulant/antiplatelet aggregation activity attributable to its hirudin sequence and integrin-directed cell attachment activity due to its RGD site. Our results suggest that this chimeric motif may serve as a prototype for a new class of anticoagulants where an integrin-specific sequence "targets" the peptide to a cell (ultimately through the platelet integrin αIIbβ3) trapped amid a thrombus with ensuing proteinase inhibition.

Hirudin is a highly specific α-thrombin inhibitor isolated from the salivary gland of the European bloodsucking leech Hirudo medicinalis (1–3). Recent structure-function studies have shown that both amino- and carboxy-terminal domains of hirudin bind to thrombin, and the isolated hirudin domains inhibit thrombin through different mechanisms (4–9). The amino-terminal hirudin domain binds to the active site of thrombin, whereas the carboxy-terminal hirudin fragment binds to the fibrinogen recognition site (adjacent to the active site) (4–9). Only a small portion of the carboxy terminus of hirudin is required for anticoagulant activity; the minimal peptide length being about 12 amino acid residues (Asn50 to Leu54) (4, 5). Hirudin and its fragments have different biochemical properties as potential therapeutic anticoagulants that could favor one over another based on the desired pharmacological characteristics.

Adhesion of blood platelets to vessel wall components and their subsequent activation is a central hemostatic event. An essential component of platelet adhesion and aggregation is the cell surface receptor αIIbβ3 (also known as glycoprotein IIb-IIIa), which is a member of the integrin family (10–14). Platelet αIIbβ3 is a receptor for four adhesive proteins: fibrinogen, fibronectin, vitronectin, and von Willebrand factor (10, 11, 13). αIIbβ3 specifically recognizes a conserved tripeptide Arg-Gly-Asp (RGD) sequence found in all four proteins and the carboxyl terminus of the γ chain of fibrinogen (HHLGGAKQAGDV) (10, 11, 13, 15). Additionally, there are many other integrins found in numerous cell types that specifically mediate both cell adhesion with substrates derived from extracellular matrix and body fluids and cell-cell interactions (10, 16).

There are examples of hybrid molecules either that combine two functions or that acquire a new function. A bifunctional thrombin inhibitor has been prepared by linking (D-Phe)Pro-Arg-Pro- and hirudin carboxy-terminal fragments (17, 18). RGD- and HHLGGAKQAGDV-containing sequences either coupled to or genetically engineered into a carrier protein have integrin-specific cell binding activity similar to the parent adhesive protein (15, 19–21). We hypothesized that an artificial chimeric peptide could be constructed that incorporated antithrombin activity and integrin-directed cell attachment activity. This chimera motif is based on the finding that platelet phospholipid microparticles produced following platelet activation contain the components for assembly of the prothrombinase complex and functional αIIbβ3 receptors (22). Therefore, coupling these sequence types into a chimera might provide a "targeted" antithrombin agent to specifically bind cells at a thrombus for inhibition of thrombin.

In the present investigation, we synthesized a chimeric peptide by adding the RGD tripeptide, a minimal cell adhesion sequence from fibronectin and other adhesive proteins, to a segment of the carboxy terminus of hirudin (termed chimeric antithrombin peptide). We report here that the chimeric antithrombin peptide has both antithrombin and cell adhesion activities comparable to its individual constituents.

EXPERIMENTAL PROCEDURES

Materials—All N-(9-Fluorenyl)methoxy carbonyl-amino acid derivatives and reagents were obtained from Milligen/Cambridge Research Biochemicals. Human α-thrombin and antithrombin were purified as described (23, 24). Heparin was provided by Diosynth; L-arginine and L-lysine were obtained from Sigma; L-leucine was obtained from Nutritional Biochemicals.
tosylamido-2-phenethyl chloromethyl ketone-treated trypsin was from Cooper Biochemicals. Bovine fibrinogen was from Miles Laboratories; Chromozyme TH (tosyl-Gly-Pro-Arg-p-nitroanilide) was obtained from Boehringer Mannheim.

**Peptide Synthesis**—Peptides were assembled using a Milligen Pep-Synthesizer as described previously (25). Purity of the peptides was analyzed by reverse-phase HPLC (24, 26), and if necessary, peptides were further purified by HPLC on a preparative Vydac C_18 column. All peptides were analyzed either by amino acid analysis or by primary structural analysis on an Applied Biosystems 475A Protein Sequencer (Protein Chemistry Laboratory, Department of Chemistry of this institution). An excellent correlation between expected and actual values/sequences was found for all peptides. Sequences of synthetic hirudin\textsuperscript{51-64} and RGD-hirudin\textsuperscript{51-64} peptides are shown below (picomoles of amino acid yield/cycle are shown in parentheses).

Hirudin\textsuperscript{51-64}: H-Asn(409)-Gly(628)-Asp(809)-Phe(645)-Glu(410)-Glu(311)-Ile(350)-Pro(257)-Glu(168)-Glu(140)-Tyr(119)-Leu(73)-Ala(29)-OH

RGD-hirudin\textsuperscript{51-64}: H-Trp(1150)-Gly(1166)-Arg(965)-Gly(715)-Asp(599)-Ser(479)-Ala(734)-Asn(508)-Gly(339)-Asp(435)-Phe(430)-Glu(263)-Glu(250)-Ile(237)-Pro(227)-Glu(154)-Glu(138)-Tyr(67)-Leu(6.4)-OH.

All other peptides and their sequences using the one-letter abbreviation (shown in parentheses) were as follows: RGD-peptide (GRODSAY, an Arg-Gly-Asp-Ser-Arg-Leu-Gly-Arg-Asp-Ser-Arg). RGE-hirudin\textsuperscript{51-64} (WGRGESANGDFEEIPEEYL), and HC\textsubscript{G39-66} (DFHKENTVTND-ologies) and antithrombin activities of synthetic peptides. Top, fibrinogen clotting activity of human α-thrombin was measured as described under "Experimental Procedures": hirudin\textsuperscript{51-64} did not affect the ability of hirudin\textsuperscript{51-64} to inhibit fibrinogen hydrolysis by thrombin (Fig. 1, top). The concentration required for 50% inhibition (IC\textsubscript{50}) for hirudin\textsuperscript{51-64}, RGD-hirudin\textsuperscript{51-64}, and RGD-hirudin\textsuperscript{51-64} was 0.6 μM. There was also a dose-dependent increase in the aPTT of normal pooled human plasma (average clotting time of 40 + 1.3 s for 100% plasma) for hirudin\textsuperscript{51-64}, RGD-hirudin\textsuperscript{51-64}, and RGD-hirudin\textsuperscript{51-64}.

**RESULTS**

**Anticoagulant and Antithrombin Activities**—Coupling the cell adhesive RGD sequence (and the inactive RGE conformation) to hirudin\textsuperscript{51-64} did not affect the ability of hirudin\textsuperscript{51-64} to inhibit fibrinogen hydrolysis by thrombin (Fig. 1, top). The concentration required for 50% inhibition (IC\textsubscript{50}) for hirudin\textsuperscript{51-64}, RGD-hirudin\textsuperscript{51-64}, and RGD-hirudin\textsuperscript{51-64} was 0.6 μM. There was also a dose-dependent increase in the aPTT of normal pooled human plasma (average clotting time of 40 + 1.3 s for 100% plasma) for hirudin\textsuperscript{51-64}, RGD-hirudin\textsuperscript{51-64}, and RGD-hirudin\textsuperscript{51-64}.

![Fig. 1. Anticoagulant and antithrombotic activities of synthetic peptides](image-url)

The abbreviations used are: HPLC, high performance liquid chromatography; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); aPTT, activated partial thromboplastin time; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.

unit/m final concentration) was added and the light transmittance (Bio/Data PAP-4 Aggregometer) was recorded. These experiments were performed in triplicate four times with four different healthy volunteers and the results averaged.

**Cells and Cell Attachment Assays**—Human dermal fibroblasts (supplied by Dr. R. A. Briggaman, Department of Dermatology of this institution) were grown in DMEM (GIBCO) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The human erethoidal cell line (EA.hy 926; supplied by Dr. C.-J. S Edgell of this institution) was grown as described previously (28). Cell adhesion activity of the synthetic peptides was determined as described (29). Briefly, -1 × 10⁵ cells/ml of trypsinized cells were mixed with DMEM containing 10% FBS and 0.5, 0.5, 1.0, 1.5, and 1.5 mg/ml of RGD, RGE, hirudin\textsuperscript{51-64}, RGD-hirudin\textsuperscript{51-64}, or RGE-hirudin\textsuperscript{51-64} peptides, respectively (these concentrations provided approximately equal molar amounts of RGD/E). Cells that attached to the microtiter plate wells after incubation for 60 min at 37 °C and 5% CO₂ were quantified by staining with Crystal Violet, solubilizing the stained cells with ethylene glycol monomethyl ether, and the absorbance at 600 nm was compared with standard curves of serially diluted cells (30). These experiments were performed from three to six times.
The presence of various amounts of heparin was performed as detailed under "Experimental Procedures" either in the absence (○) or in the presence (△) of hirudin53-64. Thrombin inhibition was determined as the second-order rate constant of inhibition (× 10⁻⁶ M⁻¹ s⁻¹).

and RGE-hirudin53-64 (Fig. 1, bottom).

We examined thrombin inhibition by the plasma serpin antithrombin in the presence of hirudin53-64 and RGD-hirudin53-64. Neither hirudin53-64 nor RGD-hirudin53-64 interfered with the thrombin-antithrombin inhibition reaction (in the absence of added heparin) as shown by second-order rate constants of 1.37, 1.22, and 1.34 (× 10⁻⁶ M⁻¹ min⁻¹) in the absence of peptide, and in the presence of a 200-fold molar excess of hirudin53-64 or RGD-hirudin53-64 to thrombin, respectively.

We also determined the effect of hirudin53-64 on thrombin inhibition by antithrombin-heparin. At a 100-fold molar excess of hirudin53-64 to thrombin, there was essentially no difference in the rate of thrombin inhibition by antithrombin in the presence of various amounts of heparin (Fig. 2).

Trypsin hydrolyzes α-thrombin at unique sites in the B-chain to form βγ- and γγ-thrombin derivatives. We assessed the effect of hirudin53-64 and RGD-hirudin53-64 on trypsinolysis of α-thrombin. Both hirudin53-64 and RGD-hirudin53-64 afforded essentially total protection to α-thrombin during incubation with trypsin (Fig. 3). Control experiments verified that the hirudin53-64-containing peptides had no inhibitory effect on trypsin.

We examined the peptides for dose-dependent inhibition of platelet aggregation in α-thrombin-stimulated human platelets. Platelet aggregation induced by α-thrombin was inhibited most effectively by hirudin53-64, RGD-hirudin53-64, and RGE-hirudin53-64 (IC50 of 7 μM for each peptide), but less effectively by the RGD-peptide (IC50 ~100 μM), and with no effect by the RGE-peptide (tested to 300 μM). Complete inhibition of α-thrombin-induced platelet aggregation was observed with 15 μM hirudin53-64, RGD-hirudin53-64, and RGE-hirudin53-64.

These data indicate that the fibrinogen clotting and platelet aggregation activities of α-thrombin (in a purified or plasma-based assay system) are inhibited to essentially the same extent by hirudin53-64 and RGD/E-hirudin53-64 and that addition of the RGD/E sequence to hirudin53-64 is not detrimental to its anticoagulant and antithrombin activities. The data also suggest that hirudin53-64 and RGD-hirudin53-64 bind to the same site on thrombin since neither influences inhibition by the plasma serpin antithrombin and both protect thrombin during trypsinolysis.

**Cell Adhesion Activity**—We compared each synthetic peptide in solution for its ability to inhibit fibroblast and endothelial cell attachment in the presence of FBS. Cell surface integrins will bind to RGD-containing adhesive proteins present in serum as FBS coats the microtiter plate surface. We found that RGD-hirudin53-64 and the RGD-peptide were quite effective at preventing cell attachment, whereas hirudin53-64, the RGE-peptide, and RGE-hirudin53-64 did not interfere with cell adhesion (Table I). Microscopic inspection of fibroblasts and endothelial cells verified that hirudin53-64, the RGE-peptide, and RGE-hirudin53-64 had no noticeable effect on cell attachment. However, RGD-hirudin53-64 and the RGD-peptide did affect adhesion in that the cells were rounded and not attached to the surface (shown for fibroblasts in Fig. 4). These data demonstrate that RGD-hirudin53-64 functions like the
RGD-peptide in inhibition of cell adhesion, but that the hirudin sequence alone does not affect cell adhesion.

We investigated whether RGD-hirudin could act as a “bridge” between RGD-specific cell receptors and thrombin (as a replacement for the adhesive proteins present in FBS). We prepared thrombin complexes with RGD-hirudin and hirudin and adsorbed the thrombin-peptide complexes to polystyrene; next, fibroblasts were added in the absence of and without synthetic peptides. We prepared thrombin complexes with RGD-hirudin and ”bridge” between RGD-specific cell receptors and thrombin. Our results with RGD-containing peptides/proteins implies that a unique sequence can be “engineered” to preferentially interact with a particular integrin (for instance, by stereochemical isomerization, cyclization, or a unique next-neighbor sequence). Indeed, RGD peptide-albumin conjugates have been shown to recognize specific integrins (19, 21), and the RGD peptide-albumin conjugates have been shown to recognize specific integrins (19, 21).

**DISCUSSION**

This study was undertaken to characterize a chimera combining RGD and hirudin sequences. Our results with RGD-hirudin are in accord with previous observations using hirudin carboxyl-terminal fragments in anticoagulant, antithrombin, and platelet aggregation inhibition assays (4–9, 32–35). Our data and those of others indicate that hirudin carboxyl-terminal fragments bind to the fibrinogen recognition site (anion exosite domain) of thrombin which effectively blocks both fibrinogen clotting and thrombin-stimulated platelet aggregation activities. These hirudin fragments also do not affect thrombin inhibition by the serpin antithrombin with or without heparin. Thus, hirudin fragments that are targeted to the anion exosite of thrombin, not the active site, may work independently of antithrombin to regulate thrombin. Future chimeric antithrombin peptide designs for the hirudin site will include variations in the sequence of the carboxyl-terminal fragment and specific chemical modification of Tyr (either by nitration, iodination, or sulfixation) in an effort to increase its overall antithrombin/anticoagulant potency.

Our results demonstrate that RGD-hirudin has the same cell-binding activity as the RGD-peptide alone; thus, RGD in the RGD-hirudin chimera must assume an active conformation. Many proteins have been identified that contain the RGD tripeptide sequence, but the presence of an RGD sequence does not necessarily confer cell adhesion activity (10). There is sufficient evidence to suggest that both RGD conformation and environment contribute to integrin-directed cell recognition (10, 13, 20, 29, 38–41). This recognition specificity (and affinity) for RGD-containing peptides/proteins implies that a unique sequence can be “engineered” to preferentially interact with a particular integrin (for instance, by stereochemical isomerization, cyclization, or a unique next-neighbor sequence). Indeed, RGD peptide-albumin conjugates have been shown to recognize specific integrins (19, 21), and the RGD peptide-albumin conjugates have been shown to recognize specific integrins (19, 21).

Our data and those of others indicate that hirudin carboxyl-terminal fragments in anticoagulant, antithrombin, and platelet aggregation inhibition assays (4–9, 32–35). Our data and those of others indicate that hirudin carboxyl-terminal fragments in anticoagulant, antithrombin, and platelet aggregation inhibition assays (4–9, 32–35). Our data and those of others indicate that hirudin carboxyl-terminal fragments in anticoagulant, antithrombin, and platelet aggregation inhibition assays (4–9, 32–35). Our data and those of others indicate that hirudin carboxyl-terminal fragments in anticoagulant, antithrombin, and platelet aggregation inhibition assays (4–9, 32–35).
target sites in these chimeras might support cooperative multifunctional activities.

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