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

(Received for publication, March 27, 1991)

Frank C. Church‡, Jeanne E. Phillips, and Joan L. Woods
From the The Center for Thrombosis and Hemostasis and Departments of Pathology and Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

We investigated the properties of an artificial chimeric peptide that contains an Arg-Gly-Asp (RGD)-
tripetide, the versatile cell recognition signal of extracellular matrix protein components, coupled to a
carboxyl-terminal fragment of the highly specific α-thrombin inhibitor, hirudin, (residues 53-64): WGRGDSDANGDFEEIPEEYL (RGD-hirudin53-64). Hirudin53-64 and RGD-hirudin53-64 inhibited the fibrino-
gen 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 antico-
gulant/antiplatelet aggregation activity attributable to its hirudin sequence and integrin-directed cell at-
tachment activity due to its RGD site. Our results suggest that this chimeric motif may serve as a proto-
type 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 protease 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 carboxyl-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 carboxyl-terminal hirudin fragment binds to the fibrinogen recognition site (adjacent to the active site) (4-9). Only a small portion of the carboxyl terminus of hirudin is required for anticoagulant activity; the minimal peptide length being about 12 amino acid residues (Asp9 to Leu64) (4, 5). Hirudin and its fragments have different bio-
chemical properties as potential therapeutic anticoagulants that could favor one over another based on the desired pharma-
cological 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: fibrino-
gen, 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 spe-
cifically mediate both cell adhesion with substrates derived from extracellular matrix and body fluids and cell-cell inter-
actions (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 carboxyl-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 incor-
porated antithrombin activity and integrin-directed cell at-
tachment 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 carboxyl terminus of hirudin (termed chimeric antithrombin peptide). We report here that the chimeric anti-
thrombin peptide has both antithrombin and cell adhesion activities comparable to its individual constituents.

EXPERIMENTAL PROCEDURES

Materials—All N-(9-Fluorenyl)methoxycarbonyl-amino acid der-
ivatives and reagents were obtained from Milligen/Cambridge Re-
search Biochemicals. Human α-thrombin and antithrombin were
purified as described (23, 24). Heparin was provided by Dionsynth; L1-

* This work was supported in part by a grant-in-aid from the North Carolina Affiliate of the American Heart Association and by a Uni-
versity Research Council grant from The University of North Caro-
olina at Chapel Hill. The costs of publication of this article were
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‡ To whom correspondence should be addressed: Campus Box 7035,
Division of Hematology, 416 Burnett-Womack, University of North
Carolina, Chapel Hill, NC 27599. Fax: 919-966-7639.

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tosylamido-2-phenethyl chloromethyl ketone-treated trypsin was from 
Cooper Biochemicals. Bovine fibrinogen was from Miles Laborat-
yories; Chromozyme TH (tosyl-Gly-Pro-Arg-p-nitroanilide) was ob-
tained 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, addi-
tional fractions were further purified by HPLC on a preparative Vydac C18 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 hirudin53-64 and RGD-hirudin53-64 peptides are shown below (picomoles of amino acid yield/cycle are shown in parentheses).

Hirudin53-64: 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-hirudin53-64: 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 abbrevia-
tion (shown in parentheses) were as follows: RGD-peptide (GROGDYSY); RGE-hirudin53-64 (WGREGSANGDFEERGL); and HC56-66 (DFHKENTVTND-
WIPEGEEDDDYLDLTEY) (25).

Anticoagulant and Antithrombin Assays—All experiments were 
performed in a buffer that contained 20 mM HEPES, 150 mM NaCl, 0.1% (w/v) polyethylene glycol (M, = 6000) at pH 7.4. Fibrinogen 
clotting activity of human α-thrombin was measured in bovine serum albumin-coated microtiter plates by incubating 50 μl of thrombin (10 
μM stock) with 50 μl of a synthetic peptide (8–20 μM stock). After 1 
min, 100 μl of fibrinogen (5 mg/ml stock) was added, briefly agitated, 
and the absorbance at 405 nm was measured every 5 s for 2 min in a 
Vmax kinetic microplate reader (Molecular Devices). These experi-
ments were performed in triplicate from three to five times. aPTT of 
pooled human plasma using Thromboscreen Kontact reagent (Pacific 
Hemostasia) was determined in the presence of synthetic peptides 
with a Fibrometer as described (27). Experiments were performed 
three times and the results averaged.

Antithrombin inhibition assay of thrombin in the presence of a 
200-fold molar excess of either hirudin53-64 or RGD-hirudin53-64 to 
 thrombin was performed as described previously (27). Thrombin 
inhibition by antithrombin-heparin in the presence of synthetic pep-
tide was performed by incubating 1 nM thrombin with 100 nM hirudin 
or 1 min, followed by 10 nM human antithrombin in the presence 
of 0.05 to 500 μg/ml heparin. After 20 s, Chromozyme TH 
with polybrene (to neutralize the added heparin) was added, substrate 
hydrolysis was stopped after 60 min by the addition of glacial acetic 
acid, and the absorbance at 405 nm was determined. Inhibition rate 
constants were calculated as detailed previously (27). These exper-
iments were performed three times.

Platelet Aggregation Assay—Platelet aggregation assays were per-
formed using human platelet-rich plasma (diluted to 300,000 plate-
lets/μl) by drawing blood (9 parts) into 3.8% (w/v) sodium citrate (1 
part) from a volunteer who had not had any aspirin or related products 
for at least 14 days. Platelet aggregation was performed by adding 40 
μl of a synthetic peptide solution to 450 μl of platelet-rich plasma at 
37 °C. After a 2-min incubation, 10 μl of α-thrombin (0.4 μM NIH 
unit/ml 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 (sup-
plied by Dr. R. A. Brigham, 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 
erotheodil cell line (EA.hy 926; supplied by Dr. C.-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 tryptsinized 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, hirudin53-64, RGD-hirudin53-64, or RGE-hirudin53-64 peptides, 
respectively (these concentrations provided approximately equal 
molar amounts of RGD/E). Cells that attached to the microtiter plate 
were assayed after incubation for 60 min at 37 °C and 5% CO2 were quantified 
by staining with Crystal Violet, solubilizing the stained cells with 
etylene glycol monomethy 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.

RESULTS

Anticoagulant and Antithrombin Activities—Coupling the cell 
adhescence RGD sequence (and the inactive RGE confor-
mation) to hirudin53-64 did not affect the ability of hirudin53-64 
to inhibit fibrinogen hydrolysis by thrombin (Fig. 1, top). The 
concentration required for 50% inhibition (IC50) for hirudin53-64, 
RGD-hirudin53-64, and RGE-hirudin53-64 was 0.4 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 hirudin53-64, RGD-hirudin53-64,

Anticoagulant and antithrombotic activities of synthetic 
peptides. Top, fibrinogen clotting activity of human α-throm-
bin was measured as described under "Experimental Procedures"; 
hirudin53-64 (□) and RGD-hirudin53-64 (●). RGE-hirudin53-64 had 
esentially the same activity as shown here for hirudin53-64 and RGD-
hirudin53-64 (data not included). A control peptide, HC56-66 (○), did 
not inhibit fibrinogen clotting activity. Bottom, aPTT assays were 
performed using normal human pooled plasma as detailed under 
"Experimental Procedures." The control HC56-66 peptide did not 
prolong the aPTT (tested at 10 μM).

Fig. 1. Anticoagulant and antithrombotic activities of synthetic 
peptides.

1 The abbreviations used are: HPLC, high performance liquid chro-
matography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic 
acid; aPTT, activated partial thromboplastin time; DMEM, Dulbec-
co's modified Eagle's medium; FBS, fetal bovine serum.
and RGE-hirudin \(^{53-64}\) (Fig. 1, bottom).

We examined thrombin inhibition by the plasma serpin antithrombin in the presence of hirudin \(^{53-64}\) and RGD-hirudin \(^{53-64}\). Neither hirudin \(^{53-64}\) nor RGD-hirudin \(^{53-64}\) 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 \(\text{M}^{-1}\text{min}^{-1}\) in the absence of peptide, and in the presence of a 200-fold molar excess of hirudin \(^{53-64}\) or RGD-hirudin \(^{53-64}\) to thrombin, respectively.

We also determined the effect of hirudin \(^{53-64}\) on thrombin inhibition by antithrombin-heparin. At a 100-fold molar excess of hirudin \(^{53-64}\) 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 \(\alpha\)-thrombin at unique sites in the B-chain to form \(\beta\)- and \(\gamma\)-thrombin derivatives. We assessed the effect of hirudin \(^{53-64}\) and RGD-hirudin \(^{53-64}\) on trypsinolysis of \(\alpha\)-thrombin. Both hirudin \(^{53-64}\) and RGD-hirudin \(^{53-64}\) afforded essentially total protection to \(\alpha\)-thrombin during incubation with trypsin (Fig. 3). Control experiments verified that the hirudin \(^{53-64}\)-containing peptides had no inhibitory effect on trypsin.

We examined the peptides for dose-dependent inhibition of platelet aggregation in \(\alpha\)-thrombin-stimulated human platelets. Platelet aggregation induced by \(\alpha\)-thrombin was inhibited most effectively by hirudin \(^{53-64}\), RGD-hirudin \(^{53-64}\), and RGE-hirudin \(^{53-64}\) (IC\(_{50}\) of 7 \(\mu\)M for each peptide), but less effectively by the RGD-peptide (IC\(_{50}\) \(~100 \mu\)M), and with no effect by the RGE-peptide (tested to 300 \(\mu\)M). Complete inhibition of \(\alpha\)-thrombin-induced platelet aggregation was observed with 15 \(\mu\)M hirudin \(^{53-64}\), RGD-hirudin \(^{53-64}\), and RGE-hirudin \(^{53-64}\). These data indicate that the fibrinogen clotting and platelet aggregation activities of \(\alpha\)-thrombin (in a purified or plasma-based assay) are inhibited to essentially the same extent by hirudin \(^{53-64}\) and RGD/E-hirudin \(^{53-64}\) and that addition of the RGD/E sequence to hirudin \(^{53-64}\) is not detrimental to its anticoagulant and antithrombin activities. The data also suggest that hirudin \(^{53-64}\) and RGD-hirudin \(^{53-64}\) 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-hirudin \(^{53-64}\) and the RGD-peptide were quite effective at preventing cell attachment, whereas hirudin \(^{53-64}\), the RGE-peptide, and RGE-hirudin \(^{53-64}\) did not interfere with cell adhesion (Table I). Microscopic inspection of fibroblasts and endothelial cells verified that hirudin \(^{53-64}\), the RGE-peptide, and RGE-hirudin \(^{53-64}\) had no noticeable effect on cell attachment. However, RGD-hirudin \(^{53-64}\) 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-hirudin \(^{53-64}\) functions like the
RGD-peptide in inhibition of cell adhesion, but that the
hirudin53-64 sequence alone does not affect cell adhesion.

We investigated whether RGD-hirudin53-64 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-hirudin53-64 and
hirudin53-64 and adsorbed the thrombin-peptide complexes to
copolyurethane; next, fibroblasts were added in the absence of
and without synthetic peptides

Our results demonstrate that RGD-hirudin53-64 has the same cell-binding activity as the RGD-peptide alone; thus, RGD in the RGD-hirudin53-64 chimera must assume an active
conformation. Many proteins have been identified that con-
tain the RGD tripeptide sequence, but the presence of an
RGD sequence does not necessarily confer cell adhesion ac-
tivity (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 recogni-
tion specificity (and affinity) for RGD-containing peptides/
proteins implies that a unique sequence can be "engineered"
to preferentially interact with a particular integrin (for in-
stance, by stereochemical isomerization, cyclization, or a
unique next-neighbor sequence). Indeed, RGD peptide-albu-
mins conjugates have been shown to recognize specific inte-
grins (19, 21).

αιβ3 is the dominant fibrinogen receptor in platelets (10-
14). Cross-linking studies have shown that RGD binds pre-
dominantly to the β3 subunit, whereas HHLGAKQAGDV
binds to the α1B subunit. There are other fibrinogen receptors
including the αιβ3 integrin found primarily on endothelial
cells (40) and αιδβ3 on leukocytes (42). Comparison of
the binding specificity of αιβ3 and α1B3 for fibrinogen shows that
αιβ3 preferentially recognizes both an RGD-peptide modeled
after Acα53-56 RGD (but not Acα572-574 RGD) and γ-chain400-411
HHLGAKQAGDV sequences, whereas α1B3 exclusively
interacts with an RGD-peptide modeled after the Acα572-574 RGD
sequence (40). There are many other integrins that interact
with different protein sites than those just described, for
instance, leukocyte αιβ3 binds a novel fibrinogen site (neither
RGD nor the carboxy terminus of the γ-chain) (42) and αιβ3
in a melanoma cell line recognizes a fibronectin sequence
consisting of X-Asp-Y (43). Therefore, it would appear that
appropriate peptide sequences can be designed to specifically
target the chimeras to platelets αιβ3 and not other integrins
able to bind fibrinogen or other proteins.

Previous studies have shown that RGD-and HHLGAKQAGDV-containing peptides prevent fibrinogen binding to
platelets and platelet aggregation and alter the conformation
of purified platelet αιβ3 (10–15). These peptide sequences
have been implicated as potential candidates for therapeutic
antiplatelet agents (13, 14). Furthermore, a family of RGD-
containing proteins from a variety of snake venoms and
leeches has recently been described as potent antiplatelet
compounds (44, 45). Future chimeric antithrombin peptide
designs for the integrin-directed site (platelet αιβ3) will in-
corporate unique/specific RGD sequences (such as that in the
fibrinogen Acα53-56 chain or that found in the snake venom
RGD-protein family) and non-RGD sequences (such as that
in the fibrinogen γ-chain400-411 sequence).

The possibility for an achievable targeted chimeric anti-
thrombin peptide is strengthened by Bode et al. (46), Sandberg
et al. (47), and more recently by the work of Sims et al. (22)
in their detection of both functional protegrhinase complex
components and αιβ3 incorporated into platelet plasma
membrane microparticles. We ultimately envision a chimeric
antithrombin peptide combining αιβ3-specific and thrombin
anion exosite-directed active sites. This peptide would be
capable of interacting with stimulated platelets trapped within
a thrombus and not only blocking platelet-fibrinogen (or other
RGD-containing proteins) interactions but also halting
thrombin-mediated fibrinogen clotting and platelet aggrega-
tion activities. Finally, the partnership of distinct/different

DISCUSSION

This study was undertaken to characterize a chimaera
combining RGD and hirudin sequences. Our results with RGD-
hirudin53-64 are in accord with previous observations using
hirudin carboxy-terminal fragments in anticoagulant, anti-
thrombin, and platelet aggregation inhibition assays (4-9, 32-
35). Our data and those of others indicate that hirudin car-
oxoy-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 throm-
bin. Future chimeric antithrombin peptide designs for the
hirudin site will include variations in the sequence of the
carboxy-terminal fragment (36) and specific chemical modi-
fication of Tyr39 (either by nitration (37), iodination (37), or
sulfation (5)) in an effort to increase its overall antithrombin/
anticoagulant potency.

Fig. 4. Effect of synthetic peptides on fibroblast adhesion.
Inhibition of fibroblast cell adhesion in the presence of DMEM/FBS
with and without synthetic peptides was performed as described under
"Experimental Procedures." a, DMEM/FBS alone; b, DMEM/FBS
and the RGD-peptide; c, DMEM/FBS and hirudin53-64; and d,
DMEM/FBS and RGD-hirudin53-64.

RGD-Hirudin Chimera

target sites in these chimeras might support cooperative multifunctional activities.

Acknowledgments—We thank Professors Charlotte W. Pratt, Mau- resne Hoffman, and Gilbert C. White, II for their helpful discussions and critical reading of the manuscript; Professors Robert A. Briga- man and Cora-Jean S. Edgell for providing the fibroblasts and endo- theelial cells, respectively; and Alicia Rico-Lazarowski (Clinical Co- ngluation Laboratory, University of North Carolina Hospitals) for assistance in the platelet aggregation studies.

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