High-affinity RNA ligands to human α -thrombin

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ABSTRACT

Systematic Evolution of Ligands by EXponential enrichment (SELEX) was used to isolate from a population of 10^{13} RNA molecules two classes of high affinity RNAs that bind specifically to human α -thrombin. Class I RNAs are represented by a 24-nucleotide RNA (RNA 16.24), and class II RNAs are represented by a 33-nucleotide RNA (RNA 27.33). RNA 16.24 inhibits thrombin-catalyzed fibrin clot formation *in vitro*. Secondary structures are proposed for these RNAs, revealing a novel stem-loop structure for RNA 16.24, comprised of an unusually large 16-nucleotide loop. Mutants of RNA 16.24 were generated to investigate structural features critical to high-affinity binding. Phosphate modification with ethylnitrosourea identified regions of the RNAs necessary for electrostatic interactions. Competition with heparin suggests that these RNAs bind the electropositive heparin-binding site of thrombin. These ligands represent a novel class of thrombin inhibitors that may be suitable for therapeutic or diagnostic applications.

INTRODUCTION

SELEX (Systematic Evolution of Ligands by EXponential enrichment) is a method developed to search among large random-sequence single-stranded oligonucleotide populations for molecules with high-affinity binding to defined molecular targets (1, 2). It has recently become apparent that RNA molecules demonstrate remarkable diversity of structure and function (3). RNAs have been isolated using this method which show high affinity and specificity for a variety of protein targets, including bacteriophage T4 DNA polymerase (1), the bacteriophage R17 coat protein (4), HIV-1 reverse transcriptase (5), and basic fibroblast growth factor (6). RNAs have been shown to bind specifically and functionally with antibiotics (7) and other simple organic molecules such as polycyclic dyes (8) and the alkaloid theophylline (9). An RNA (ribozyme) capable of catalyzing a nucleotide ligation reaction has also been reported (10). The diversity of structure and function of single-stranded DNA molecules is also being explored using the SELEX technology. Single-stranded DNAs that bind to several targets have been identified and may be compared with the RNA ligands to these targets (8, 11, and manuscripts in preparation).

A particularly interesting protein target for SELEX is thrombin, a pivotal enzyme in the regulation of thrombosis and hemostasis. Thrombin is a multifunctional serine protease exhibiting both procoagulant and anticoagulant functions. As a procoagulant enzyme thrombin cleaves fibrinogen, activates clotting factors V, VIII, and XIII, and activates platelets (12, 13). As an anticoagulant enzyme, thrombin binds to thrombomodulin (TM), altering thrombin substrate specificity from fibrinogen and platelets to the anticoagulant zymogen protein C, through a combination of an allosteric change in the active site conformation and an overlap of the TM and fibrinogen binding sites on thrombin (14).

The thrombin protein is composed of various structural domains, including the catalytic site and two electropositive domains, a fibrinogen-recognition exosite and a heparin-binding site (15 and refs therein). The specificity of thrombin lies in binding of substrate (fibrinogen, thrombin receptor) or cofactors (TM) to the fibrinogen-recognition exosite (16, 17, 18). Inactivation of thrombin by plasma antithrombin III (ATIII) requires simultaneous binding of heparin to thrombin and ATIII to form a termolecular complex (19 and refs therein). The most potent and specific known inhibitor of thrombin is the leech protein hirudin (20). Hirudin owes its high affinity ($K_d=10^{-14}$) and specificity to bivalent binding, as it interacts with both the catalytic site and the fibrinogen-recognition exosite (15, 18, 21, 22, and refs therein).

While thrombin is a protein with no known nucleic acid binding function, the SELEX strategy has been used to identify two different RNAs that bind with high affinity. One of these RNAs inhibits fibrin clot formation *in vitro*. The predicted structural features of these RNAs and their interaction with thrombin are described. Independently, a single-stranded DNA that binds thrombin has also been described (23). Structural and functional differences between the RNA and DNA ligands are discussed.

MATERIAL AND METHODS

SELEX

A population of 10^{13} DNA templates with a 30-nucleotide (30N) variable region and 5' and 3' fixed regions was created by oligonucleotide synthesis and PCR (5, 6). The 5' primer containing the T7 promoter (underlined) with the sequence 5'-CCGAAGCTTAATACGACTCACTATAGGGAGATGCC-

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TGTCGAGCATGCTA-3', and the 3' primer with the sequence 5'-CCGTCGACAAAGCTGTTTAGCTAC-3' were used with a single-stranded DNA template containing a 30N variable region, 5'CCCGTCGACAAAGCTGTTTAGCTAC[30N]CAGCATGCTCGACAGGCATCT-3' to create a 104-nucleotide double-stranded PCR product. Random RNA molecules used for the selections were generated by *in vitro* transcription from the T7 promoter sequence in the 5' end of the double-stranded DNA template (2).

Equimolar concentrations of human α -thrombin protein (Sigma, Enzyme Research Laboratories) and RNA (approximately 5×10^{-7} M) were used for selection in a 100 μ l reaction mixture containing 50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 1 mM DTT, and 1 mM $MgCl_2$ (binding buffer) for 5 min at 37°C. Each binding reaction was filtered through a nitrocellulose filter (2.5 cm Millipore, 0.45 μ m) pre-wetted with wash buffer (50 mM Tris-HCl, pH 7.7) in a Millipore filter binding apparatus, and immediately rinsed with 5 ml wash buffer. The protein-bound RNA was extracted from the filters in 400 μ l phenol and 200 μ l freshly prepared 7 M urea. The RNA was precipitated with 20 μ g tRNA, and was used as a template for cDNA synthesis, followed by PCR and *in vitro* transcription to prepare RNA for the subsequent round (1, 4, 5). RNA was prefiltered through nitrocellulose filters (1.3 cm Millipore, 0.45 μ m) before the 3rd, 4th, 5th, 8th, 11th, and 12th rounds to diminish any nonspecific nitrocellulose filter binding.

The equilibrium dissociation constants (K_d s) of the evolving RNA population for thrombin were determined after the 5th, 8th, and 12th rounds. These experiments were performed in protein excess at concentrations from 2.0×10^{-6} to 5.0×10^{-10} M at a final RNA concentration of 5×10^{-11} M. Transcribed RNA for these binding curves was radiolabeled with [α - 32 P]ATP. Binding of the thrombin-RNA complex to nitrocellulose filters was as described for the rounds of SELEX, except that the filter-bound RNA was dried and quantitated by scintillation counting.

Following the 12th round of SELEX, the RNA was sequenced with reverse transcriptase (AMV, Life Sciences, Inc.) using the 32 P-5'-end-labeled 3' complementary PCR primer (above).

Cloning and sequencing RNAs

RNA from the 12th round was reverse transcribed to DNA and amplified by PCR. The DNA was digested at restriction enzyme sites *Hind*III and *Sal*I at the 5' and 3' ends, respectively, and ligated into the complementary sites in the *E. coli* cloning vector pUC18 (4). Ligated plasmid DNA was transformed into *E. coli* JM103 cells and transformants were identified by blue/white colony formation. Colonies containing unique sequences were grown up and double-stranded plasmid DNAs were used for dideoxy sequencing with the Sequenase kit version 2.0 (United States Biochemical) and [35 S]dATP (Amersham). RNA was transcribed from cloned plasmid DNA as above with T7 RNA polymerase (New England Biolabs, Inc.) (24).

Determination of 5' and 3' boundaries

RNA from individual clones with determined sequences was 32 P-labeled at the 5' and 3' ends for determination of 3' and 5' boundaries, respectively. For 5' end-labeling, *in vitro* transcribed RNA was dephosphorylated at the 5' end with alkaline phosphatase (calf intestinal, Boehringer-Mannheim), and 5' end-labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (New England Biolabs, Inc.). RNA was labeled at the 3'-end with [α - 32 P]pCp by T4 RNA ligase (New England BioLabs, Inc.),

30 min, 37°C. 5' and 3' end-labeled RNAs were purified on an 8% polyacrylamide gel containing 8 M urea.

Alkaline hydrolysis of RNAs 3' or 5' end-labeled for the 5' or 3' boundary experiments, respectively, was carried out in a 10 μ l reaction containing 50 mM sodium carbonate, pH 9.0, 1 mM EDTA for 10 minutes at 90°C. The reaction was stopped by precipitation of the RNA. Binding reactions containing 40, 10, or 2.5 nM thrombin and approximately 0.5 pmoles hydrolyzed RNA were performed in thrombin binding buffer (above) for 5 min at 37°C. Protein-bound RNA was isolated by nitrocellulose filter binding and RNA was extracted as described above. RNA fragments were electrophoresed on a 12% sequencing gel. Unbound hydrolyzed RNA was run as a control. To provide the hydrolyzed RNA with a sequence orientation, an RNase T1 digest of the labeled RNA, which specifically hydrolyzes RNAs at the 3' phosphate of guanosine residues, was electrophoresed alongside the unbound and bound hydrolyzed RNA. The T1 digest was carried out for 10 min at 50°C in a 10 μ l reaction containing 500 fmoles RNA and 5 units RNase T1 (Boehringer-Mannheim) in buffer containing 7 M urea, 20 mM sodium citrate, pH 5.0, and 1 mM EDTA. The reaction was terminated by adding 10 μ l stop dye and stored at -20°C for electrophoresis.

Synthetic RNA and DNA

RNA/DNA molecules were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer using Millipore phosphoramidites, according to manufacturer's specifications.

Ethyl nitrosourea (ENU) modification of phosphates

5'-End-labeled RNA (5–10 pmoles) was incubated with ethanol saturated ENU (10 μ l) in a 50 μ l reaction mixture containing 50 mM Tris-HCl, pH 7.7, 1 mM EDTA at 92°C for 5 min. Under these conditions, the kinetics of alkylation are approximately a single phosphate per RNA molecule. Alkylated RNA (1–2 pmoles) was bound to thrombin at 90, 30, 10 and 5 nM in thrombin binding buffer (above) at 37°C for 5 min. Protein bound RNA, isolated by nitrocellulose filter binding, was extracted from filters (above) and precipitated with tRNA as a carrier, air dried and resuspended in 10 μ l 100 mM Tris-HCl, pH 9. RNA was hydrolyzed at 50°C for 7 min. Stop dye was added to the mixture on ice, and hydrolysis bands were resolved on a 15% sequencing gel. An RNase T1 digest of unmodified RNA and a hydrolysis ladder of unbound modified RNA were run alongside the lanes with bound, modified RNA to orient the sequence and to show kinetics of ENU modification and hydrolysis.

Competition assays

32 P-end-labeled RNAs 16.24 or 27.33 (100 nM) and increasing concentrations of heparin (Sigma) (10^{-10} to 10^{-3} M) or the sulfated C-terminal dodecapeptide of hirudin [Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(OSO₃)-Leu] (American Diagnostica), referred to here as hir53-64(SO₄), (10^{-10} to 10^{-4} M) were incubated under binding conditions described above. Thrombin (50 nM) was added to the mixture, incubated at 37°C for 5 min and thrombin-bound RNA was partitioned by nitrocellulose filter binding.

Similarly, for competition between RNAs, 32 P-end-labeled RNAs 16.24 and 27.33 (100 nM) and increasing concentrations of cold competitor RNA 16.24 or 27.33 (10^{-10} to 10^{-5} M) were incubated under binding conditions described above.

Amidolytic assay of thrombin activity

Thrombin hydrolysis of the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-pNitroaniline) (Chromogenix) was measured photometrically at 405 nm due to the release of p-nitroaniline. Thrombin was added to a final concentration of 10^{-8} or 10^{-9} M to a reaction buffer (50 mM sodium citrate, pH 6.5, 150 mM NaCl, 0.1% PEG), containing 250 μ M S2238 substrate at 37°C. For inhibition assays, thrombin plus RNA (equimolar or 10-fold excess) were preincubated 30 sec at 37°C before adding to the reaction mixture.

Fibrinogen clotting

The effect of the RNAs 16.24 and 27.33 on clotting time was measured with a fibrometer at 37°C. Human fibrinogen (generous gift, Michael Mosesson, Sinai Samaritan Medical Center, Milwaukee, WI or Enzyme Research Laboratories) at a concentration of 2 mg/ml plus 1 unit RNasin (Promega) was incubated in thrombin binding buffer containing 0.1% PEG. RNA 16.24 or 27.33 was added at 2, 20, 200 or 1000 nM. Thrombin (1 NIH unit) was added to a final volume of 200 μ l. Alternatively the RNA and thrombin were preincubated before adding to the fibrinogen mixture with equivalent results. To control for nonspecific inhibitory effects of RNA, a 30-nucleotide polyadenosine (A30) non-binding RNA or a mutated sequence (5'-CCGGAUCGAAGUUAGUAGGCCGAG-3') of RNA 16.24 (5'-UCCGGAUCGAAGUUAGUAGGCCGA-3') which no longer binds with high affinity ($K_d > 10^{-6}$), were included in the reaction at 1000 nM.

RESULTS

RNA sequences and dissociation constants

A pool of 10^{13} RNA molecules with a variable region of 30 nucleotides (30N) was used to isolate high-affinity RNA ligands to thrombin using SELEX (1). The DNA template and primers

used to generate the double-stranded template for transcription are described in Methods. Sequencing of the RNA population following 12 rounds of SELEX revealed a nonrandom population. Subsequent rounds did not improve the affinity of the RNA population for thrombin. RNA from the 12th round was reverse transcribed, amplified, and cloned. The sequences of 28 individual molecules were determined revealing two highly conserved sequence motifs, designated class I and class II (Figure 1). Class I RNAs were the most abundant, representing 22 of 28 sequences, while class II RNAs represented 6 of 28 sequences. Of the 22 sequences of class I, 16 (8 of which were identical) contained an identical sequence motif: GGAUCGAAG(N)₂A-GUAGGC, whereas the remaining 6 contained 1 or 2 nucleotide changes in the defined region or some variation in N=2 to N=5. This conserved motif varied in its position within the 30N region. In class II, 3 of 6 RNAs were identical and all of them contained the conserved motif GCGGCJUUGGGCGCCGUGCUU, beginning at the 3rd nucleotide from the end of the 5' fixed region.

Affinities of individual full-length RNA clones (variable 30N region flanked by 5' and 3' fixed sequences) within each class were determined by nitrocellulose filter binding as described above. K_d s were determined by the least squares fit of the data points to a binding equation that assumes a simple bimolecular RNA-thrombin interaction (6). Clones 16 and 27 represented RNAs from class I and class II with the lowest K_d s; approximately 37 ± 3.5 nM and 114 ± 2.0 nM, respectively. The binding curves for RNAs 16 and 27 and the random 30N population are shown in Figure 2.

RNAs 16 and 27 were shown to bind specifically to thrombin and not to the abundant serum proteins ATIII, prothrombin and fibrinogen. ATIII is also a heparin binding ($K_d=20$ nM) protein (19), prothrombin is the biochemical precursor to thrombin, and fibrinogen is the primary thrombin substrate in clot formation. These ligands did not bind at protein concentrations as high as

	5'fixed	30N variable	3'fixed
Class I (22 clones)			
#1,6,13,19, 23,24,25,30	gggagaugccugucgagcaugcug	<u>AGGATCGAAG</u> UUU <u>AGUAGGC</u> UUUGUGUGCUC	guagcuaaacagcuuugucgacggg
#2	gggagaugccugucgagcaugcug	UACUG <u>GAATCGAAG</u> GU <u>AGUAGGC</u> CAGUCAC	guagcuaaacagcuuugucgacggg
#5	gggagaugccugucgagcaugcug	AUAUCAC <u>GGATCGAAG</u> GAA <u>AGUAGGC</u> GUG	guagcuaaacagcuuugucgacggg
#9	gggagaugccugucgagcaugcug	CCUUCCCG <u>GUUCGAAG</u> UCC <u>AGUAGGC</u> CGG	guagcuaaacagcuuugucgacggg
#10	gggagaugccugucgagcaugcug	CACCC <u>GGATCGAAG</u> UUU <u>AGUAGGC</u> GUGAGU	guagcuaaacagcuuugucgacggg
#15	gggagaugccugucgagcaugcug	UGUAC <u>GGATCGAAG</u> GU <u>AGUAGGC</u> CAGUUAC	guagcuaaacagcuuugucgacggg
#16	gggagaugccugucgagcaugcug	CA[UCC <u>GGATCGAAG</u> UUU <u>AGUAGGC</u> GGGA]GUG	guagcuaaacagcuuugucgacggg
#18	gggagaugccugucgagcaugcug	AUUGUUGC <u>GGATCGAAG</u> UGG <u>AGUAGGC</u> CGCA	guagcuaaacagcuuugucgacggg
#21	gggagaugccugucgagcaugcug	UGUACU <u>GGATCGAAG</u> GU <u>AGUAGGC</u> CAGUCAC	guagcuaaacagcuuugucgacggg
#22	gggagaugccugucgagcaugcug	<u>GAATCGAAG</u> UUU <u>AGUAGGC</u> AGCGUGUG	guagcuaaacagcuuugucgacggg
#26	gggagaugccugucgagcaugcug	ACGCUGGAGUC <u>GGATCGAAG</u> AGGUA <u>AGUAGGC</u> GACU	guagcuaaacagcuuugucgacggg
#31	gggagaugccugucgagcaugcug	GGGUC <u>GGATCGAAG</u> AGGUA <u>AGUAGGC</u> GACU	guagcuaaacagcuuugucgacggg
#33	gggagaugccugucgagcaugcug	AUAUCAC <u>GGATCGAAG</u> AGAG <u>AGUAGGC</u> GU	guagcuaaacagcuuugucgacggg
#34	gggagaugccugucgagcaugcug	UGUACU <u>GGATCGAAG</u> GU <u>AGUAGGC</u> AGGCAC	guagcuaaacagcuuugucgacggg
#37	gggagaugccugucgagcaugcug	AUAUCAC <u>GGATCGAAG</u> GAA <u>AGUAGGC</u> GUG	guagcuaaacagcuuugucgacggg
Class II (6 clones)			
#3	gggagaugccugucgagcaugcug	GUG <u>CGCGC</u> UUU <u>GGGCGCCG</u> CGUGGC	guagcuaaacagcuuugucgacggg
#20	gggagaugccugucgagcaugcug	GUG <u>CGCGC</u> UUU <u>GGGCGCCG</u> CGUGAC	guagcuaaacagcuuugucgacggg
#27,38,39	gggagaugccugucgagcaugcug	GUG <u>CGCGC</u> UUU <u>GGGCGCCG</u> CGUGGAC	guagcuaaacagcuuugucgacggg
#35	gggagaugccugucgagcaugcug	GG <u>CGCGC</u> UUU <u>GGGCGCCG</u> CGUGAC	guagcuaaacagcuuugucgacggg

Figure 1. Nucleotide sequences of RNA ligands. Individual sequences are grouped as class I or class II and aligned by conserved sequence motifs, indicated by bold, underlined characters. Each sequence is divided into 3 blocks from left to right. 1) the 5'-fixed region, 2) the 30N variable region, and 3) the 3'-fixed region. Brackets indicate 5' and 3' boundaries on RNA clones 16 and 27.

Figure 3. Proposed secondary structures of truncated RNA ligands. The 24-nucleotide class I RNA from clone 16, designated 16.24, and the 33-nucleotide class II RNA from clone 27, designated 27.33, and their Kds are shown. The 5' and 3' fixed regions are depicted by lower case lettering, the 30N random region by upper case lettering and the conserved regions are in bold face lettering and are underlined. Asterisks indicate alkylated phosphates required for thrombin binding at all thrombin concentrations (see Figure 5).

3. From these experiments, it is possible to map phosphate interactions to the loop of RNA 16.24 and to the stem region of RNA 27.33 (with a single modified nucleotide in the loop showing some interference). In both cases the critical phosphate residues lie in regions within the conserved domains of these RNAs.

Given the polyanionic properties of RNA, the selected ligands might bind to the electropositive domains of thrombin, either the fibrinogen-recognition exosite or the heparin-binding site. Competition for binding to thrombin with heparin or hir53-64(SO₄) (the C-terminal domain of hirudin which binds competitively to the fibrinogen-recognition exosite) (18) was used to map the interaction of these RNAs to either of these sites. The results of these competition experiments are shown in Figure 6A. Data were fit using a standard competition equation and fitting algorithm, assuming a 1:1 stoichiometry (29). The apparent competitor dissociation constant (K_c) for heparin is 0.78 μ M for RNA 16.24 and 0.94 μ M for RNA 27.33. The K_d for heparin binding to thrombin ranges from 0.86–2.4 μ M (19, and refs

therein). Under conditions where the RNA and competitor bind to the same site, K_c equals K_d . As the K_c s for RNAs 16.24 and 27.33 are close to the K_d for heparin binding to thrombin, these data indicate that these RNAs bind to or overlap the heparin binding site. In contrast to heparin, there was virtually no competition for binding of RNA 16.24 ($K_c=210 \mu$ M) or RNA 27.33 ($K_c=390 \mu$ M) with hir53-64(SO₄). As hir53-64(SO₄) inhibits procoagulant activity of thrombin with a K_i of approximately 150 nM (18), these results indicate that the RNAs are not binding the fibrinogen-recognition exosite.

In order to determine whether these RNAs are binding to the same site on thrombin, or whether their binding was mutually exclusive, competition experiments were performed in which the RNAs were allowed to compete with themselves or alternatively with each other for binding. The results of these competition assays are shown in Figure 6B. In each case, the RNA was able to compete for its own binding with a K_c that was nearly equivalent to the K_d . RNA 16.24 was able to compete for binding of RNA 27.33 with a $K_c=K_d$, but RNA 27.33 was unable to completely compete for binding of RNA 16.24. These results suggest that these RNAs bind to overlapping sites on thrombin.

Figure 4. RNA 16.24 mutants and dissociation constants (K_d s). The proposed secondary structures of wild-type class I RNA 16.24 and mutants 16.24-G, 16.24-GC, and 16.25-GC are shown. Conserved nucleotides are in bold face lettering and are underlined. The K_d s for thrombin binding, as determined by nitrocellulose filter binding are indicated.

Figure 5. ENU alkylation of phosphates and thrombin binding. Autoradiogram showing sites (asterisks) where ENU alkylation of phosphates interfered with thrombin binding. The T1 digest of end-labeled RNA is shown in lane 1), the hydrolysis ladder of unbound modified RNA in lane 2), binding of modified RNAs with 90 nM thrombin in lane 3), 30 nM thrombin in lane 4), 10 nM thrombin in lane 5), and 5 nM thrombin in lane 6). Double asterisks indicate alkylated phosphates lost only at low (5 nM) protein concentration where these contacts become increasingly important. Indicated phosphates are shown on the predicted secondary structure diagram of Figure 3.

Functional assays

The hydrolysis by thrombin of the chromogenic substrate S-2238 was measured in the presence and absence of RNA. There was no inhibitory effect on this cleavage reaction (data not shown). Since this tripeptide is specific for the catalytic site these results suggest that the RNA ligands are not binding in the catalytic site of thrombin, nor is its binding affecting the ability of this tripeptide substrate to be cleaved by the thrombin enzyme.

The effect of the RNA ligands 16.24 and 27.33 on fibrin clot formation as measured by a fibrometer are shown in Figure 7. These results show that RNA 16.24 has an inhibitory effect on the conversion of fibrinogen to fibrin and clot formation, with an IC_{50} of approximately 200 nM. RNA 27.33 had little effect on this conversion. Neither the 30-nucleotide polyadenosine A30 RNA nor a mutated RNA 16.24 had any effect on clot formation at 1000 nM (data not shown). To confirm the specificity of this inhibition, these assays were repeated with Reptilase® (American Diagnostica), a snake venom enzyme known to form a fibrin clot by cleavage of fibrinopeptide A from fibrinogen. None of the inhibitors had any effect on this reaction, confirming that the RNA 16.24 inhibitor was thrombin specific (data not shown).

DISCUSSION

The data reported here represents the use of the SELEX method to isolate two classes of specific high-affinity RNA ligands to thrombin. The class I sequences are represented by a 24-nucleotide RNA, designated 16.24. The class II sequences are represented by a 33-nucleotide RNA, designated 27.33. The K_d s for RNA 16.24 and RNA 27.33 are approximately 9 nM and 155 nM, respectively.

The Zucker RNA folding algorithm (25) identifies stem-loop structures for both RNAs. The proposed secondary structure model for RNA 16.24 is unique, however, in that it comprises a loop structure of 16 nucleotides. Mutation of RNA 16.24 resulting in a 15 nucleotide loop structure, without altering the conserved nucleotides, resulted in an RNA that no longer bound with high affinity to thrombin (Figure 4). These results suggest that binding of RNA 16.24 is not due strictly to recognition of the conserved linear nucleotide sequence, but that this sequence contains information through interactions with base, ribose or phosphate groups to provide secondary and tertiary structural stability. Enzymatic probing of the structures of these RNAs with

Figure 6. Competition binding analysis. In A) competition for binding of ^{32}P -end-labeled RNAs 16.24 and 27.33 by heparin (top) or hir53-64(SO_4) (bottom). In B) competition for binding of ^{32}P -end-labeled RNA 16.24 by unlabeled RNA 16.24 or 27.33 (top) and competition for binding of ^{32}P -end-labeled RNA 27.33 by unlabeled RNA 16.24 or 27.33 (bottom). The data were normalized by dividing the % RNA bound in the presence of increasing concentrations of competitor by the % bound in the absence of competitor to give % Bound shown on the abscissa.

specific RNases confirms the proposed stem-loop structures. Further analysis of the solution structures are being investigated with chemical probes and by NMR analysis (manuscript in preparation).

Differences in protein binding between RNAs 16.24 and 27.33 were also evident by phosphate modification interference assays. ENU alkylation of RNA may result in steric-related misfolding of RNA or binding interference but is very often used to map phosphates that are necessary for electrostatic interactions with protein. Phosphate residues throughout the loop of RNA 16.24 are required for thrombin binding (Figure 3). In contrast, phosphates on either side of the upper portion of the stem and perhaps a nucleotide of the loop of RNA 27.33 are required for thrombin binding.

Mapping the binding site of these RNA ligands to thrombin could be inferred from several experiments. Competition for binding of RNAs 16.24 and 27.33 with heparin, and lack of competition for binding by hir53-64(SO₄) suggests that these RNAs bind to the highly electropositive heparin-binding site and not the fibrinogen-recognition exosite. In addition, the amidolytic cleavage of a small tripeptide substrate by thrombin was not inhibited by RNA 16.24 or 27.33, suggesting that they do not bind in the catalytic site of the enzyme. Furthermore, competition between these RNA ligands suggests that they bind to adjacent overlapping sites.

Thus, while RNA 16.24 and 27.33 differ in their affinities, predicted structural features and in their physical interaction with thrombin, they apparently bind to overlapping sites within the heparin-binding site of thrombin. The differences in the physical interactions of RNAs 16.24 and 27.33 with thrombin might explain why RNA 16.24, but not RNA 27.33, inhibited fibrin clot formation *in vitro*. Furthermore, the results suggest that RNA 16.24 binds not to the substrate-binding site, but rather the heparin-binding site and that inhibition of clotting is a result of noncompetitive inhibition. A noncompetitive mode of inhibition is also suggested by the plateau in inhibition seen at a competitor concentration of approximately 200 nM.

It is interesting that the putative hairpin-loop structures of these RNA ligands are quite different from the G-quartet structure

formed by a single-stranded DNA (ssDNA) ligand to thrombin (23, 30, 31). The putative binding sites on thrombin for the RNAs and the ssDNA are also different. In solution, the RNAs are likely to bind to the electropositive heparin-binding site of thrombin while the ssDNA binds to the electropositive fibrinogen-recognition exosite (32, 33). These sites are known to be spatially distinct. As expected, there is no competition for binding between the RNA and DNA ligands (data not shown).

The strongest electrostatic field on thrombin is observed for the heparin-binding 'groove', while the 'convex' thrombin surface which is the fibrinogen-recognition exosite presents the second region of high positive charge density (15, 34). The differences in binding sites between the RNAs and the ssDNA ligand could be related to the structures of these ligands, hairpin versus G-quartet. The structures of these ligands may in turn be related to the selection conditions. Potassium (5 mM) used in the selection buffer for the 15-mer ssDNA, but not for the RNA, may have enabled formation of the G-quartet structure in solution as suggested by NMR analysis (30, 31). Co-crystallization of the 15-mer ssDNA with thrombin also revealed a G-quartet structure for the DNA (32). However, in this analysis the crystals were grown in 0.19 M NaCl, in the absence of potassium. Alkali cations such as potassium and sodium have both been shown to stabilize guanine quartets in RNA and DNA structures (35 and refs therein). The RNA selection buffer contained 100 mM NaCl, an adequate concentration for G-quartet formation. We are currently investigating the relative roles for potassium and sodium ions in generating the G-quartet structure of oligonucleotide ligands for thrombin and their importance to inhibitory functions. Structural preferences between RNA and DNA ligands, independent of cation requirements, however, may become apparent as these ligands are selected to a wide range of protein targets by SELEX.

A clinical need exists for better anticoagulants. Heparin, the only approved rapid-acting thrombin inhibitor, has a number of shortcomings, among which are inaccessibility to fibrin-bound thrombin (36, 37) thrombocytopenia and bleeding. The RNA ligands described here can be modified to resist degradation in serum (38). The potency of these inhibitors can be increased by linking the RNA to known thrombin catalytic site inhibitors (39). Alternatively, these sequences can be used as a molecular anchor to select for a larger RNA with more extensive protein interactions, including the active site. One can also envision development of a heparin mimic that could be more specific for the ATIII-mediated inhibition of thrombin. It is clear from this work and related work (40) that oligonucleotides have great potential as antithrombotic agents.

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Figure 7. Fibrinogen cleavage and clot formation. The *in vitro* conversion of fibrinogen to fibrin and resulting clot formation was measured with a fibrometer in the presence of increasing concentrations (2, 20, 200, and 1000 nM) of class I RNA 16.24 and class II RNA 27.33. Clotting times are the average of at least 3 experiments.

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