Screening of functional antidotes of RNA aptamers against bovine thrombin

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Abstract A specific RNA aptamer (T705) against bovine thrombin had been obtained after seven rounds of SELEX (systematic evolution of ligands by exponential enrichment) selection from a random RNA library previously. In order to further investigate the relationship between the structure and function of this aptamer, three truncated RNA aptamers, T705a, T705b and T705c, were designed according to the secondary structure of T705 RNA. Our results showed that T705c keeping the precise stem–loop structure but lacking most of the stem region sequence of T705 could inhibit clot formation in vitro in the same way as its parental form. We also report here that single-stranded DNA (ssDNA) antisense oligonucleotides, c′ and c′-22, which were complementary to different portions of T705c could act as efficient antidotes reversing the inhibitory activity of T705. It is demonstrated for the first time that ssDNA antisense oligonucleotides are potential antidotes of RNA aptamers and this may be an effective, rapid strategy to find antidotes of RNA aptamers which would be of important usefulness in basic research and drug screening.

Key words: Systematic evolution of ligands by exponential enrichment; RNA aptamer; Antisense single-stranded DNA; Antidote; Bovine thrombin

1. Introduction

Bovine thrombin is widely used in clinical wound healing after surgery. There is 85% homology between bovine thrombin and human thrombin, so most antibodies against bovine thrombin cross-react with human thrombin. Rare antibodies against bovine thrombin that do not cross-react with human thrombin have been reported. The SELEX (systemic evolution of ligands by exponential enrichment) combinatorial chemistry methodology has been extensively used in screening for high-affinity and specific aptamers binding to the targets [1–4]. We performed in vitro selection techniques to screen a nucleic acid-based combinatorial library of about 10^{14} species for RNA aptamers capable of binding and inhibiting bovine thrombin-catalyzed fibrin clot formation [5]. Two aptamers that could bind bovine thrombin specifically and did not cross-react with human thrombin were obtained. Secondary structure prediction revealed that one of the aptamers, T705 binding bovine thrombin with higher affinity (dissociation constant $K_d = 168$ nM), had potential stem–loop secondary structure. In this report, we investigated the relationship between the secondary structure of aptamer T705 and its biological activity in vitro with different truncated T705 RNA aptamers, and also found for the first time that single-stranded DNA (ssDNA) antisense oligonucleotides could be potential antidotes of RNA aptamers.

2. Materials and methods

2.1. Generation of truncated RNA aptamers

Three truncated RNA aptamers derived from aptamer T705 were generated by in vitro transcription. Briefly, ssDNA templates were first synthesized and then amplified by polymerase chain reaction (PCR) with the following sequences:

T705a: 5′-TAATACGACTCACTATAGCAATGGTACGGTACTTCCTTTGGAAGATAACCAAAAGTGCACGCTACTTTGCTAA-3′; T705b: 5′-TAATACGACTCACTATAGCAATGGTACGGTACTTCCAAAAGCTGGAGAACTTTTCAAAAGTGCACGCTACTTTGCTAA-3′; T705c: 5′-TAATACGACTCACTATATTTGGAAGATAGCTGGAGAACTAACCAAA-3′ (the underlined sequences are T7 promoter sequences). PCR products were purified on 8% native polyacrylamide gel electrophoresis (PAGE) gel and then used as templates for in vitro transcription, which was performed with the RiboMAX™ RNA production system (Promega) from the T7 promoter sequence in the 5′ end of the double-stranded DNA template. The RNA products were purified on 7 M urea 8% denaturing PAGE gel and then used for fibrinogen clotting assay.

2.2. Fibrinogen clotting assay

The effects of three truncated RNA aptamers on clotting were assessed by measuring the time required for clotting with a fibrometer as described previously [5]. Truncated RNAs or T705 RNA (100 nM each) and thrombin (0.2NIH) were pre-incubated before being added to the fibrinogen mixture. Bovine fibrinogen (Sigma) at a concentration of 2 mg/ml in binding buffer containing 0.1% polyethylene glycol and 1 unit of RNasin (Promega) was added to the reaction with a final volume of 200 μl.

2.3. Generation of the ssDNA antisense oligonucleotides

The ssDNA antisense oligonucleotides were synthesized and purified by SBS Genetech. The sequences of these oligonucleotides were 5′-TTTG GTT AGT TCT CCA GCT ATC TTC CAA ATA TAG TGA GTC GTA TTA-3′ (c′), 5′-AGT TCT CCA GCT ATC TTC CAA A-3′ (c′-22), 5′-CCA GCT ATC TTC CAAA-3′ (c′t) and 5′-TTG GTT AGT TCT-3′ (c′-12) respectively. The effects of these synthesized ssDNA oligonucleotides were tested by fibrinogen clotting assay 15 min after adding the oligonucleotides to the aptamer–bovine thrombin complex mixture.

2.4. Gel shift assays

Gel shift assay was performed as described previously [5]. RNA

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T705 (0.5 pmol) labeled with -α-^{32}P]UTP was incubated for 15 min with varying concentrations of the synthesized ssDNA antisense oligonucleotides in binding buffer before loading on a native 8% polyacrylamide gel at 4°C. The gel was exposed to X-ray film at −80°C.

3. Results

3.1. Minimal sequence determinations

The aptamer T705 is a 68 nt RNA molecule with a potential stem–loop secondary structure analyzed by the RNA structure 3.71 (Microsoft). The top two loops of T705, loop 1 and loop 2, were considered as the functional structures of T705. Three truncated RNA molecules were designed according to the secondary structure of T705. Of these, T705a lacked loop 2, T705b lacked loop 1, and T705c possessed both loop 1 and loop 2 but lacked most of the stem region of T705 (Fig. 1). The fibrinogen clotting assay results showed that T705c had the same inhibitory effect on the conversion of fibrinogen to fibrin and clot formation as T705. But the truncated aptamers T705a and T705b had no significant effects on clot formation (Fig. 2).

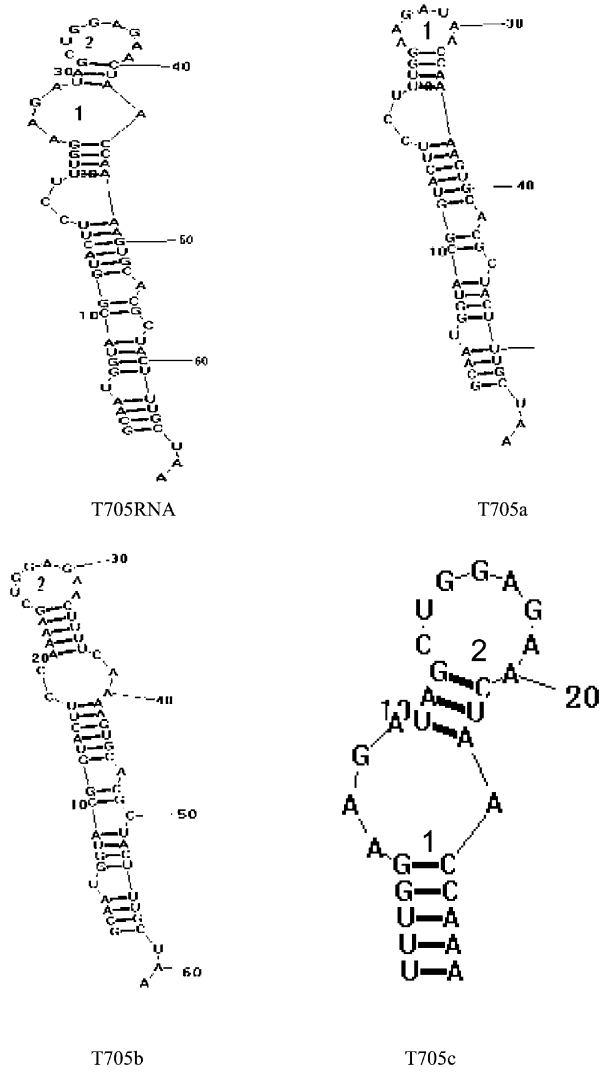


Fig. 1. Proposed secondary structures of aptamer T705 and its truncated forms T705a, T705b, and T705c. The aptamer T705 is a 68 nt RNA molecule with a potential stem–loop secondary structure analyzed by the RNA structure 3.71 (Microsoft). The top two loops of T705, loop 1 and loop 2, were considered as the functional structures of T705. Three truncated RNA molecules were designed according to the secondary structure of the T705. Of these, T705a lacked loop 2, T705b lacked loop 1, and T705c possessed both loop 1 and loop 2 but lacked most of the stem region of T705.

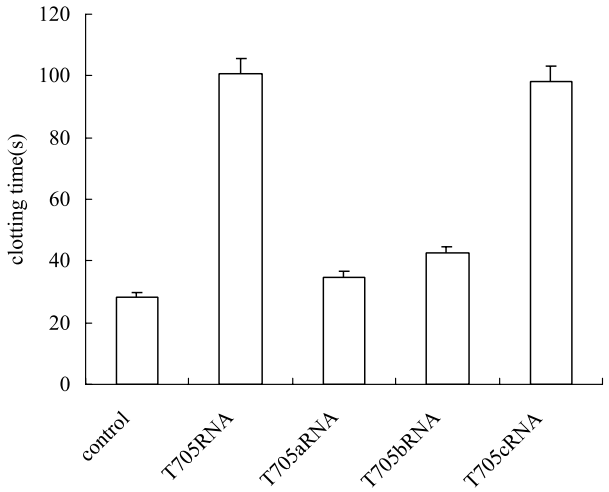


Fig. 2. Effects of aptamer T705 and its truncated forms on fibrinogen cleavage and clot formation. The same inhibition effect on the conversion of fibrinogen to fibrin and clot formation was observed with the truncated aptamer T705c as with its parental form T705. But the truncated aptamers T705a and T705b had no significant effects on clot formation.

3.2. The reversed activity of the ssDNA antisense oligonucleotides

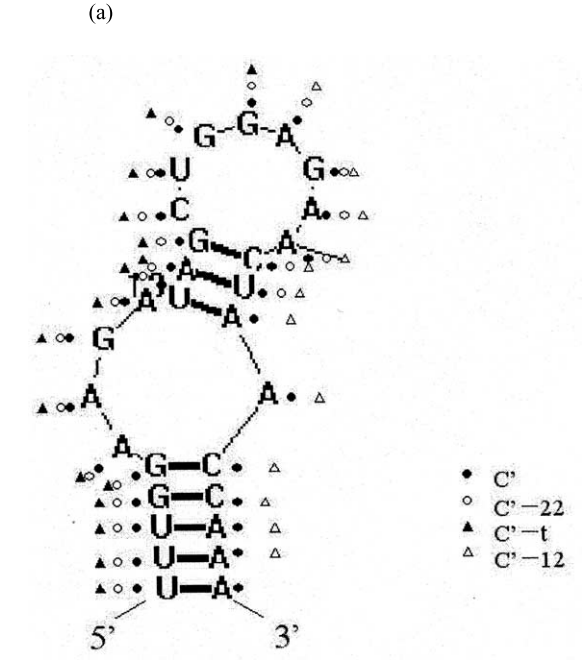
A series of ssDNA antisense oligonucleotides complementary to the different portions of aptamer T705c were designed as shown in Fig. 3a. The results showed that significant reversed clot inhibition activity of T705 was observed after adding ssDNA antisense oligonucleotides c′ or c′-22 to the aptamer–bovine thrombin complex mixture for 15 min, while the oligonucleotides c′t and c′12 had no such effects (Fig. 3b). The results also showed that the inhibition effect of oligonucleotide c′ on the clot formation of T705 was dose-dependent (Fig. 3c).

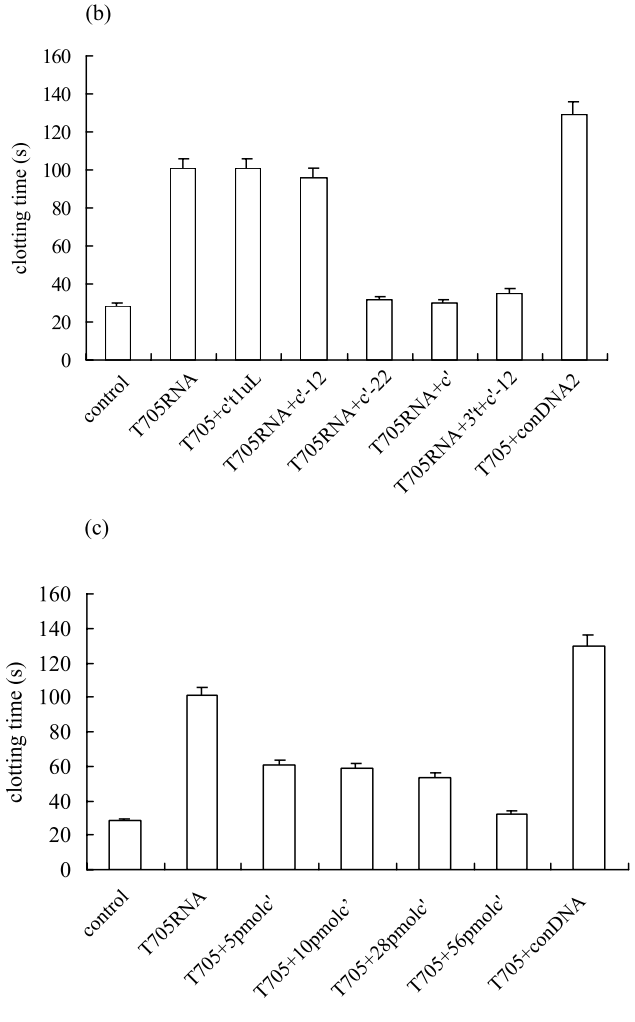
3.3. Binding ability of the antidote c′ to RNA aptamer T705 in vitro

Gel shift results showed that the functional antidote of T705, ssDNA antisense oligonucleotide c′, could bind RNA T705 in vitro. The increase in the amount of ssDNA/RNA complex was also dose-dependent (Fig. 4), similar to the inhibition effect of oligonucleotide c′ on the clot formation of T705 observed above.

4. Discussion

Specific RNA aptamers against bovine thrombin could be obtained by SELEX technology [5]. In order to further investigate the relationship between the secondary structures of aptamer T705 and its inhibition activity to bovine thrombin, we designed three truncated RNA aptamers according to the secondary changes of T705. The proposed secondary structures showed that they could fold as exactly as what we expected. We found that removal of the fixed flank sequence of RNA T705 had no effect on its inhibitory activity. However,





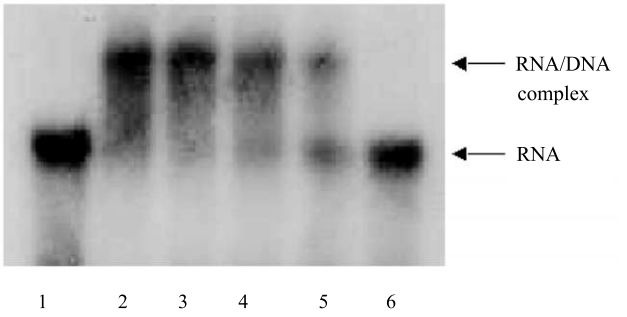


Fig. 4. Native gel analysis of antidote oligonucleotides binding to aptamer T705. Lane 1: free [^{32}P]UTP-labeled T705 RNA; lanes 2–5: [^{32}P]UTP-labeled T705 RNA was incubated with oligonucleotide c′ in different molar ratios of 1:4, 2:1, 4:1, 8:1 individually; lane 6: scrambled control ssDNA oligonucleotides incubated with [^{32}P]UTP-labeled T705 RNA. RNA aptamer T705 (0.5 pmol) labeled with [α-^{32}P]UTP was incubated for 15 min with varying concentrations of the synthesized ssDNA antisense oligonucleotide c′ in binding buffer before loading on a native 8% polyacrylamide gel at 4°C. The gel was exposed to X-ray film at −80°C.

lack of any sequences of random region of RNA T705 could lead to the elimination of its specific binding activity to bovine thrombin, which might be caused by changes of its secondary structure. This demonstrates that the precise stem–loop structure is the basic structure for the aptamer to exert its inhibitory activity.

The ability to rationally design antidotes by altering the conformation and activity of aptamers is unique to aptamers. Rusconi and his colleagues have successfully taken complementary RNA oligonucleotides as antidotes capable of efficiently reversing the anticoagulant activity of RNA aptamers against coagulation factor IXa in the clinic. They recommended a safer regulating therapeutics based upon aptamer–antidote pairs for drug action [6]. Compared with the RNA antidotes, the ssDNA antidotes have advantages, such as easy synthesis and much more stable DNA molecules. We designed four antisense ssDNA oligonucleotides complementary to different portions of T705c as potential antidotes. The clotting time assay indicated that oligonucleotides c′ and c′22 could completely reverse the anticoagulant activity of T705 in 15 min and shorten the clotting time in a dose-dependent manner, while oligonucleotides c′t and c′12 could not. The reason might be that the pairing between c′t and T705 or c′12 and T705 is not strong enough to destroy the secondary structure of T705. The pairing of at least 22 bases from T705 loops could contribute substantially to the overall binding free energy, and alter the secondary structure of the aptamer leading

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Fig. 3. Designing of antidote oligonucleotides to aptamer T705 RNA. a: Antidote oligonucleotides were designed according to the second structure of T705c: c′, which is complementary to the aptamer T705c (filled circles); c′22, which is complementary to 22 bases of the aptamer T705c 5′ end (open circles); c′t, which is complementary to 16 bases of the aptamer T705c 5′ end (filled triangles); and c′12, which is complementary to 12 bases of the aptamer T705c 3′ end (open triangles). b: Reversal effects of oligonucleotide c′, c′12 and c′22 (250 pmol) on clot formation of aptamer T705 (18 pmol). c: Dose-dependent inhibition effect of oligonucleotide c′ (5, 10, 28, and 56 pmol) on clot formation of aptamer T705 (18 pmol).

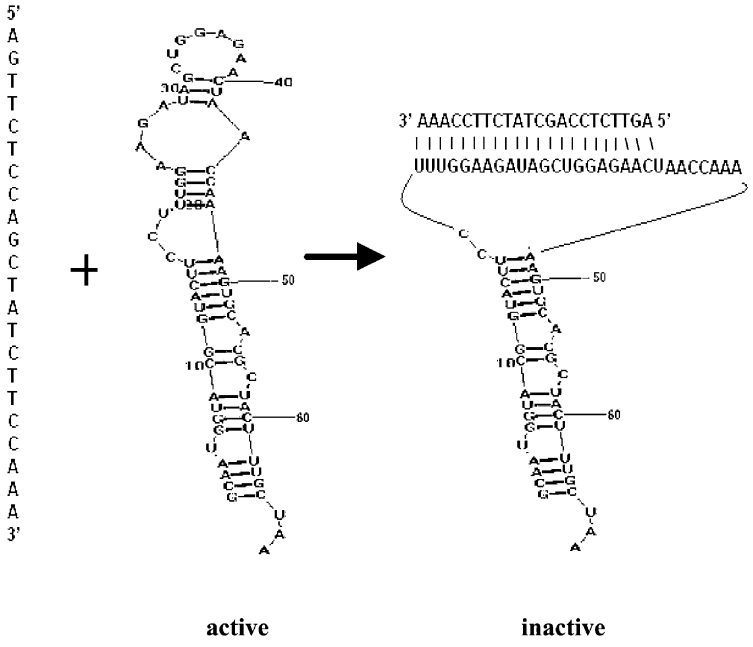

Fig. 5. Cartoon depicting the antidote oligonucleotide c′ binding to aptamer T705. The antidote oligonucleotide c′ could bind to aptamer T705 to form an aptamer–antidote complex and cause the loss of biological activity of aptamer T705.

to T705 changing from an active conformation to an inactive conformation (Fig. 5). In this paper, we also show for the first time that ssDNA antisense oligonucleotides are potential antidotes of RNA aptamers. Our paper shows an effective, rapid approach to get durable antidotes of RNA aptamers that would be of important usefulness in basic research and drug screening.

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