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The Selection of DNA Aptamers for Two Different Epitopes of Thrombin Was Not Due to Different Partitioning Methods

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Nearly all aptamers identified so far for any given target molecule have been specific for the same binding site (epitope). The most notable exception to the 1 aptamer per target molecule rule is the pair of DNA aptamers that bind to different epitopes of thrombin. This communication refutes the suggestion that these aptamers exist because different partitioning methods were used when they were selected. The possibility that selection of these aptamers was biased by conflicting secondary structures was also investigated and found not to contribute. The preparation of protein-coated magnetic beads for systematic evolution of ligands by exponential enrichment (SELEX) and the different specificities of the thrombin aptamers for the α and β forms of thrombin are also reported.

Introduction

Aptamers are high affinity recognition molecules with applications in diagnostics, therapy, and separation science. They are isolated by screening chemically synthesized combinatorial libraries of 10^{12}–10^{16} oligonucleotides for sequences that bind to a chosen target molecule (Stoltenburg et al., 2007; Famulok et al., 2007; Cho et al., 2009). The screening process known as SELEX consists of repeated cycles of *in vitro* selection and polymerase chain reaction (PCR) amplification. Nearly all aptamers so far identified for a given target molecule are specific for the same binding site (epitope). The most notable exception to the 1 aptamer per target molecule rule is the pair of DNA aptamers that bind to different epitopes of thrombin (Bock et al, 1992; Tasset et al., 1997). These aptamers (herein referred to as APT-15 and APT-29) have become the focus of more follow-up research than all other aptamers combined, and therefore it is important to gain a better understanding how they were discovered. One suggestion is that these aptamers owe their existence to the different partitioning methods that were used when they were discovered. This communication shows that the choice of partitioning method would not have prevented the discovery of both aptamers in the original work on thrombin aptamers.

Materials and Methods

Oligonucleotides (except Apt-29 inserted between the Bock primers), thrombin from human plasma (product number T7572), albumin from human serum (HSA), gelatin from cold-water fish skin and concanavalin A (Con A)-sepharose were from Sigma. The Apt-29 inserted between Bock primers sequence was from Eurofins MWG Operon. Antibodies were from Abcam. Two batches of rabbit polyclonal secondary antibody to sheep immunoglobulin G (product number: ab96946) were purchased (lot number GR62446-2 received March 2011, and lot number GR30517-1 received January 2012). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on 12% Tris-HEPES nUView Precast Gels (Generon, Maidenhead, UK) at 150V for 30 minutes. Gels were blotted onto a 0.45-μm pore size nitrocellulose membrane filter paper sandwich in NuPAGE Transfer Buffer (both from Invitrogen) containing 10% methanol at 25 V for 2 hours. Before incubation with aptamers or antibodies, nitrocellulose membranes were blocked overnight in phosphate-buffered saline (PBS) containing 5% w/v gelatin. Ultraviolet/visible spectra were recorded on a Hewlett Packard 8452A Diode Array Spectrophotometer. Fluorescence images were acquired with GenePix 4100A Personal Microarray Scanner. Slow-tilt rotation was carried out with a Dynal/Invitrogen MX2 sample mixer.

Thrombin was biotinylated by dissolving each vial of as-supplied ERL (Enzyme Research Laboratories, Swansea, UK) thrombin in 50 μL of molecular grade water to give a 3.43 mg/mL solution in 0.1 mM sodium citrate buffer, pH 6.5, containing 0.4 M NaCl and 0.2% polyethylene glycol 8000 (PEG-8000). A molar equivalent of EZ-Link NHS-PEG4-Biotin [PEG-biotin; molecular weight (MW) = 588.67; Thermo Scientific] in 5 μL of dry DMSO was added to the solution with gentle mixing, followed immediately by 50 μL of 2M sodium bicarbonate solution; the bicarbonate solution increases the

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pH to 8.0 and initiates aminolysis of the NHS. After gentle mixing for 1 hour, biotinylated thrombin was purified on a 7k MWCO Zeba spin column (Thermo Scientific) with 50 mM sodium citrate buffer, pH 6.5, containing 0.2 M NaCl as the eluting buffer. The concentration of thrombin in the eluate was determined using an extinction coefficient of E_{280} 1% = 18.3. HSA was biotinylated in the same way. MyOne streptavidin magnetic beads (Invitrogen) were washed in HEPES buffer (20mM HEPES, 150mM NaCl, 2mM KCl, 2mM, $MgCl_2$, 2mM $CaCl_2$, pH 7.4) and slow-tilt rotated for 1 hour with biotinylated thrombin at the rate of 40 μg of thrombin per mg of beads. At the end of this time the beads were washed with HEPES. Magnetic beads coated with HSA were prepared in the same way. PEG-biotin beads were prepared by rotating beads with PEG Biotin that had previously been incubated in 1 M bicarbonate solution to hydrolyze the NHS ester.

The beads were validated by slow tilt rotating 25 μg of thrombin magnetic beads in 1mL of PBS for 30 minutes with anti-thrombin antibodies (polyclonal raised in sheep) in 1 mL of PBS-Tween (15 mM sodium phosphate, 0.15 M NaCl, 0.05% Tween-20, pH 7.4) at the rate of 10 μg of antibodies per 25 μg of beads. At the end of this time the beads were washed 3 times with PBS-Tween and then rotated for 30 minutes with anti-sheep antibodies (polyclonal raised in rabbit) labeled with DyLight 649 in PBS-Tween at the rate of 10 μg of antibodies per μg of beads. At the end of this time the beads were washed 3 times with PBS-Tween, removing all solution after the final wash. The beads were then resuspended in 10 μL of water, and 1 μL volumes were spotted onto glass microscope slides. After allowing the spots to dry, the slides were imaged with a microarray scanner. Aptamer oligonucleotides were thermally conditioned before use by heating to 95°C for 5 minutes, cooling to 4°C, and allowing them to attain room temperature in HEPES buffer. Twenty-five micrograms of thrombin magnetic beads in 1mL HEPES buffer were slow-tilt rotated for 30 minutes with cyanine 5 (Cy5)-labeled aptamer. At the end of this time the beads were washed with 4 × 1 mL of HEPES buffer, removing all solution after the final wash. The beads were then resuspended in 10 μL of water, and 1 μL volumes were spotted onto a microscope slide and imaged.

Results and Discussion

The first DNA aptamer for thrombin (APT-15) was isolated by Bock and colleagues from a DNA library with the formula **5′-CGTACGGTCGACGCTAGCN_{60}GGATCCGAGCTCC ACGTG,** where the sequences in bold are for PCR priming, and N_{60} is a 60-base combinatorial sequence (Bock et al., 1992). Five cycles of selection were carried out with thrombin supplied by Sigma attached to concanavalin A-agarose (Con A-agarose) as the separation phase. Analysis of the products identified an aptamer (APT-15 in Fig. 1) that binds to exosite 1 of thrombin. Two other groups later identified aptamers composed of APT-15 with additional bases that formed a terminal duplex (APT-15-QD in Fig. 1) (Macaya et al., 1993; Wang et al., 1993). One of these groups concluded that the dominance of exosite 1 precluded the isolation of aptamers for other epitopes (Wang et al., 1993). Two years later a DNA aptamer for a different epitope of thrombin was isolated by Tasset and colleagues from a library with the general formula **5′-AGATGCCTGTCGAGCATGCT$N_{30/60}$GTAGCTAAACT GCTTTGTCGACGGG**, where $N_{30/60}$ is a 30- or 60-base

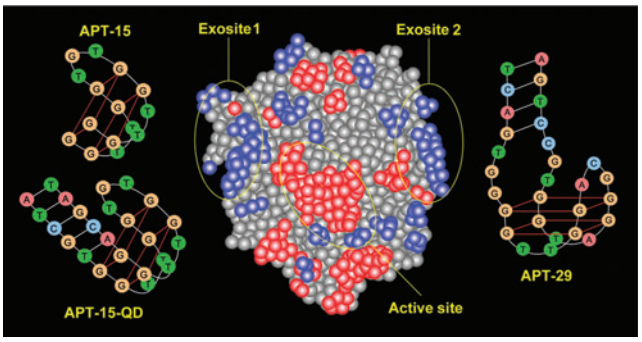

FIG. 1. Human α-thrombin and the DNA aptamers that bind to it. G-quadruplexes in the aptamers are indicated by guanine bases (G) connected by red lines. Color images available online at www.liebertpub.com/nat

combinatorial sequence (Tasset et al., 1997). The library was incubated with thrombin supplied by ERL for 5 minutes, followed by nitrocellulose filter binding to separate thrombin from unbound DNA. Analysis of products from the eleventh cycle identified an aptamer (APT 29 in Fig. 1) that binds to exosite 2 of thrombin.

Our interest in the thrombin aptamers was prompted by our investigation of SELEX using next-generation sequencing. For this we require a separation phase for DNA bound to thrombin; magnetic beads were chosen because of previous experience with this separation phase (Wilson et al., 2003; Wilson et al., 2007a; Wilson et al., 2007b; Wilson et al., 2009; Wilson, 2011). To avoid steric hindrance we prepared beads with the structure shown in Fig. 2. For counter-selection we attached HSA to beads in the same way. To validate the beads we interrogated them with antibodies from sheep and stained them with fluorescent second antibodies from rabbits. The results in Fig. 3 show that HSA beads were only stained by anti-HSA antibodies (column 2), but beads coated with thrombin supplied by Sigma were stained by both interrogation schemes (columns 1 and 2). Three hypotheses that

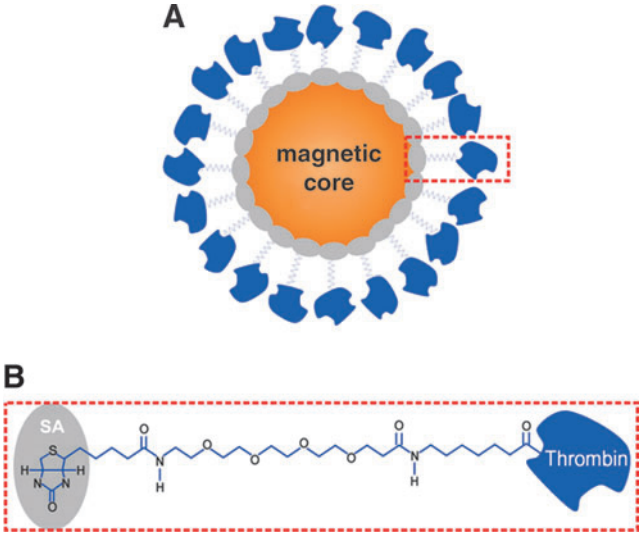

FIG. 2. (A) Streptavidin magnetic beads coated with thrombin. **(B)** Mode of attachment of biotinylated PEG-thrombin to streptavidin. PEG, polyethylene glycol. Color images available online at www.liebertpub.com/nat

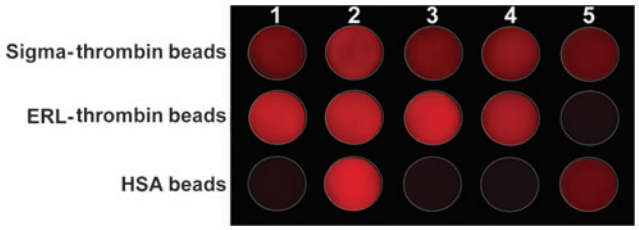

FIG. 3. Interrogation of beads with antibodies. Lane 1, anti-thrombin followed by fluorescent anti-sheep; lane 2, anti-human serum albumin (HSA) followed by fluorescent anti-sheep; lane 3, fluorescent anti-sheep only; lane 4, fluorescent anti-thrombin only; lane 5, fluorescent anti-HSA only. Color images available online at www.liebertpub.com/nat

would account for this are (1) the thrombin was contaminated with HSA; (2) the antibodies were not specific; (3) the thrombin was contaminated *and* the antibodies were not specific. Investigation of these hypotheses led to a better understanding of why there are 2 DNA aptamers for thrombin.

Electrophoresis (Fig. 4) shows that the Sigma thrombin has a well-defined band at the same MW (67 kDa) as HSA; there are also bands at 70, 130, and 240 kDa, and several larger than 300 kDa. The presence of contaminating proteins means that the beads are not suitable for *in vitro* selection. Electrophoresis of HSA shows that it also contains other proteins, but none have MWs corresponding to thrombin (or prothrombin that also binds to APT-15) (Kretz et al., 2006), and therefore they are suitable for counter-selection. MS (Fig. 5) confirmed that the Sigma product contained HSA. This is an important observation because of the continuing widespread use of Sigma thrombin in aptamer research (for example, Lee et al., 2008; Yang et al., 2009; Miyachi et al., 2010; Diculescu et al., 2011; Hoon et al., 2011; Wang et al., 2010).

Bock (Bock et al., 1992) used thrombin supplied by Sigma to select APT-15 but provided no additional information. Sigma now supplies 7 grades of human thrombin but was unable to provide information about what it supplied when Bock and colleagues carried out their work. The thrombin supplied by Sigma is graded by activity, and the grade used by us had an activity of 400–1000 units per mg. There is no suggestion in the supplier's product information that it contains HSA and other proteins, and the extinction coefficient for pure thrombin provided is unhelpful. Bock and colleagues used an unusual partitioning method in which thrombin was bound to concanavalin A-agarose (Con A-agarose) as a separation phase. The reason given was previous problems of non-specific binding with thrombin covalently attached to agarose, and the observation that thrombin was a glycoprotein. HSA is not a glycoprotein, but there is no suggestion that Bock intended to eliminate contaminating HSA. The use of Con A, however, might have allowed them to work with pure thrombin. To investigate this we purified Sigma thrombin using Con A affinity chromatography. Electrophoresis (Fig. 4, Lane 5) shows this removes contaminating HSA, but other bands remain, suggesting that if the thrombin used by Bock was impure, their separation phase would still have been contaminated with other proteins.

Tasset and colleagues (Tasset et al., 1997) used thrombin supplied by ERL. This company informed us that their product has not changed since the work of Tasset. Electrophoresis produces an intense band at the same MW as thrombin and a less intense band at 27 kDa (which is also present in the Sigma product before and after Con A chromatography) that increases with time. Peaks corresponding to HSA and other proteins are absent, and MS confirms that thrombin is nearly 100% of the total. We speculated that the 27-kDa band was β-thrombin, which is produced by autolytic cleavage of α-thrombin. Electrophoresis of thrombin solutions stored at room temperature showed that the intensity of the 27-kDa band increased with time. β-thrombin does not have an intact exosite 1 (Fitter and James 2005), and therefore APT-15 should have a lower affinity for it. Blots interrogated with fluorescent APT-15 and APT-29 (Fig. 4) show that APT-29 stains both α-thrombin and the β-thrombin band, but APT-15 only stains α-thrombin. To our knowledge this is the first time that the ability of APT-15 to discriminate between the α and β forms of thrombin has been demonstrated.

Interrogation of biotinylated ERL thrombin with fluorescent streptavidin (Fig. 4) showed that both the α and β forms

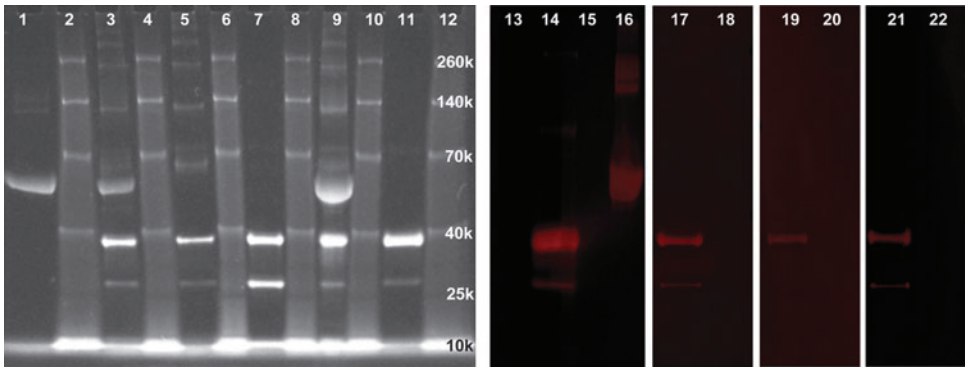

FIG. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis: lane 1, HSA; lane 2 molecular weight ladder (MWL); lane 3, Sigma thrombin; lane 4, MWL; lane 5, Sigma thrombin after Con A affinity chromatography; lane 6, MWL; lane 7, ERL thrombin; lane 8, MWL; lane 9, biotinylated Sigma thrombin; lane 10, MWL; lane 11, biotinylated ERL thrombin; lane 12, MWL. Western blots interrogated with fluorescent streptavidin lanes: 13 ERL thrombin; 14 = biotinylated ERL thrombin; 15 = HSA; 16 = biotinylated HSA. Western blots interrogated with fluorescent anti-sheep antibodies: lane 17, ERL thrombin; lane 18, HSA. Western blots interrogated with APT-15: lane 19, ERL thrombin; lane 20, HSA. Western blots interrogated with APT-29: lane 21, ERL thrombin; lane 22, HSA. Color images available online at www.liebertpub.com/nat

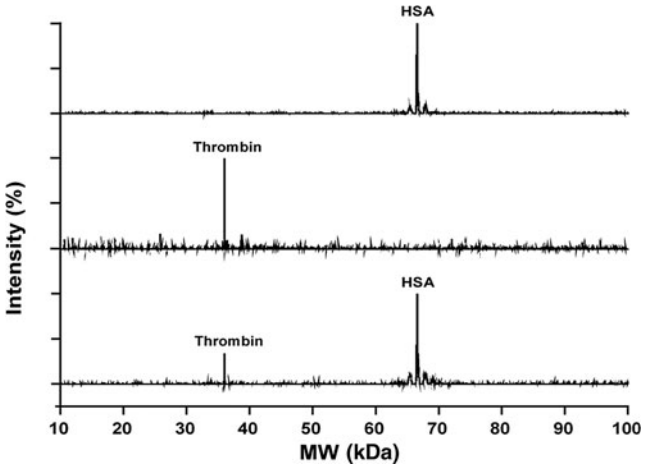

FIG. 5. Mass spectrograms. Black line, Sigma thrombin; red line, ERL thrombin; blue line, HSA.

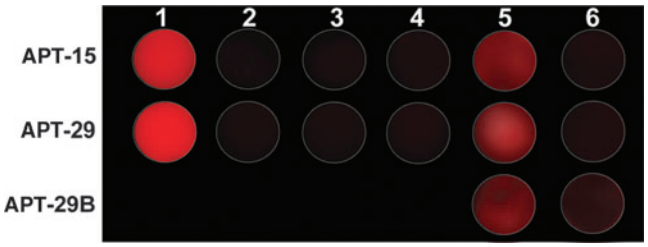

FIG. 6. Interrogation of beads and concanavalin A (Con A)-sepharose with Cy5-labeled APT-15, APT-29, and APT-29 inserted between the Bock primer sequences (APT-29B). Lane 1, Enzyme Research Laboratories (ERL) thrombin beads; lane 2, HSA beads; lane 3, PEG beads; lane 4, streptavidin beads; lane 5, ERL thrombin immobilized on Con A-sepharose; lane 6, Con A-sepharose. Color images available online at www.liebertpub.com/nat

were biotinylated, but interrogation of the corresponding beads showed that they were still stained by both antibody schemes (Fig. 3). To investigate this we interrogated the beads with anti-thrombin and anti-HSA antibodies labeled in-house with a fluorescent dye, and with as-supplied fluorescent anti-sheep antibodies. Interrogation with in-house labeled antibodies correctly discriminated between beads coated with ERL thrombin and beads coated with HSA, but interrogation with anti-sheep antibodies gave a positive result with the thrombin beads; the latter result was verified by purchasing a new batch of antibodies. Antibodies are widely perceived to be more specific than aptamers, but the results reported here show that thrombin aptamers are specific for their target, while commercial antibodies for rabbit immunoglobulin G are unable to discriminate between their cognate target and human thrombin. Figure 6 shows APT-15 and APT-29 correctly discriminate between beads coated with thrombin and HSA. Similar interrogation of beads coated with streptavidin and unconjugated biotin-PEG showed no significant binding to components other than thrombin. Autolysis of thrombin attached to beads is a potential problem, but interrogation of beads rotated at room temperature for up to a week, or stored for up to six weeks at 4°C, revealed no significant decrease in affinity for APT-15 or APT-29.

Tasset and colleagues (Tasset et al., 1997) suggested (and others have suggested since) that the isolation of DNA aptamers for different epitopes of thrombin may have been due to different partitioning methods. Bock used Con A-agarose, and Tasset used nitrocellulose-filter binding. Tasset suggested steric hindrance may have prevented potential aptamers from gaining access to exosite 2 of thrombin bound to Con A. We immobilized thrombin on Con A-agarose using the same conditions as Bock and interrogated it with fluorescent APT-15 and APT-29. Results in Fig. 6 show both aptamers bind to thrombin immobilized in this way. If APT-29 was present in the library screened by Bock, it would have been bracketed by additional bases and 2 primer sequences. Analysis of APT-29 inserted between the Bock primers reveals 3 potential hairpins that potentially interfere with conserved bases at the 5′-end of APT29 as shown in Fig. 7. This suggests a possible explanation for why Bock failed to identify APT-29, but it is difficult to predict what effect competition between these hairpins and other secondary structures would have on APT-29. To clarify this we interrogated thrombin-Con A with a fluorescent version of this oligonucleotide (APT-29B) using the same conditions as Bock. Results in Fig. 6 show that it does bind, but there is a higher background signal in the absence of thrombin.

In conclusion, we have shown that the existence of 2 different aptamers for thrombin is not due to the unusual partitioning method used by Bock. The selection of APT-29 by Bock would not have been prevented by steric hindrance as suggested by Tassett. In fact, when both aptamers are incubated with the thrombin bound to Con A at the same concentration, APT-29 gives the more intense signal, which implies that its selection would have been favored by this partitioning method. An alternative reason for the failure of Bock to discover APT-29 is disruption of its structure by the Bock primer sequences. Secondary structure analysis reveals the presence of potential hairpins, but experimental investigation shows that these do not prevent binding of APT-29 to thrombin. This leaves unanswered the ultimate question of why there are 2 aptamers for thrombin. We suspect that the solution to this mystery is related to the small library used by Bock, which may have had a diversity of less than 10^{12}, and the tendency of aptamers to converge on a single epitope after many rounds of selection (Fitter and James 2005), such as the 11 rounds applied by Tasset. We are currently investigating these factors using next generation sequencing and hope to show how they contributed to the discovery of the thrombin aptamers in the near future.

5′-**CGTACGGTCGACGCT**AGCCAGTAATAGACACTAACA**GTCCGTGGTAGGGCAGGTTGGGGTGACT**CA
TACAAGCTCATCA**CACGTGGAGCTCGGATCC**

5′-**CGTACGGTCGACGCT**AGCCAGTAATAGACACTAAC**AGTCCGTGGTAGGGCAGGTTGGGGTGACT**CA
TACAAGCTCATCA**CACGTGGAGCTCGGATCC**

5′-**CGTACGGTCGACGCT**AGCCAGTAATAGACACTAAC**AGTCCGTGGTAGGGCAGGTTGGGGTGACT**CA
TACAAGCTCATCA**CACGTGGAGCTCGGATCC**

FIG. 7. Potential hairpins when APT-29 is bracketed by Bock primer sequences. APT-29 is inserted into a 97-base sequence (the same length as used by Bock). The Bock primer sequences and APT-29 are shown in bold type. The other bases do not contribute additional hairpins. Bases that potentially form hairpins are shown with underscore.

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Author Disclosure Statement

No competing financial interests exist.

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