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IN VITRO SELECTION OF PHOSPHOROTHIOLATED APTAMERS

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Abstract: A pool of RNA molecules that contained exclusively phosphorothioate internucleoside linkages was used as a starting point for the selection of aptamers that bind to basic fibroblast growth factor (bFGF), and appear to act as heparin mimics.

Introduction

Aptamers that bind tightly and specifically to ligands can be selected from random sequence populations of nucleic acids.[1,2] However, since natural nucleic acids are inherently unstable in many biological milieu, such as sera, their potential effectiveness as therapeutic or diagnostic reagents may be limited. In order to stabilize nucleic acids to enzymatic degradation, modified nucleotides have been introduced into random sequence pools prior to selection and into selected binding species (aptamers) following selection.[3] In particular, RNA pools that contained 2′ modified pyrimidines have been used to identify aptamers that can bind tightly and specifically to vascular endothelial growth factor (VEGF), bFGF, and keratinocyte growth factor (KGF).[4–6] Modified nucleotides can also be added following selection. For example, caps containing deoxynucleotides with phosphorothioate linkages were appended to both ends of an anti-VEGF aptamer and stabilized the aptamer to exonuclease degradation.[4]

However, preselection substitutions have so far been largely limited to pyrimidine nucleotides, primarily because of the synthetic difficulties inherent in purine nucleotide chemistry. In order to generate more fully substituted aptamers than those that have previously been studied, we have selected RNA molecules that can bind to bFGF from a random sequence population in which every position in the backbone contained a phosphorothioate rather than a phosphate.

Materials and Methods

Materials: Human recombinant bFGF was purified from yeast expressing an artificial bFGF cDNA, (Zymogenetics, Seattle, WA) as described previously.[7] Phosphorothioate nucleotide triphosphates were purchased from NEN Life Sciences Products (Boston, MA).

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In vitro selection: Standard procedures for in vitro selection or SELEX experiments have been described and were followed throughout.[8] Phosphorothioate containing RNA (PSRNA) transcripts were prepared from DNA templates using an Ampliscribe T7 in vitro transcription kit (Epicentre Technologies, Madison, WI). The transcription reactions proceeded for 6 h at 42 °C and PSRNA was then purified on 10% polyacrylamide gels containing 7 M urea. Full-length transcripts were excised, eluted from the gel by soaking in 0.3 M NaCl overnight, and ethanol precipitated. PSRNA pellets were resuspended in 20 μL TE and quantified spectrophotometrically. The PSRNA samples were then diluted in PBS, phosphate buffered saline (101 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) pH 7.4, heat denatured at 65 °C, renatured at room temperature for 10 min, and passed over a nitrocellulose filter to remove filter-binding species. After 1 h of incubation with bFGF at room temperature, protein-bound PSRNA was partitioned from the rest of the pool by nitrocellulose filtration. Protein:PSRNA complexes were eluted from the filter by boiling in elution buffer (7 M urea, 100 mM sodium citrate pH 5.0, 3 mM EDTA, 0.5 mM ß-mercaptoethanol) for 5 min. Selected PSRNA was precipitated with isopropanol, resuspended in water, and amplified in a 100 μL RT-PCR reaction (0.2 mM dNTPs, 5% acetamide, 0.05% Nonidet-P40, 0.5 μM of the 3′ primer (Figure 1), 0.5 μM of the 5′ primer (Figure 1), 30 mM Tricine (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, 0.5 U *Taq* polymerase (Promega, Madison, WI), and 1U *AMV* reverse transcriptase (Seikagaku, Ijamsville, MD)). The DNA generated from this amplification was ethanol precipitated and used for the next round of selection.

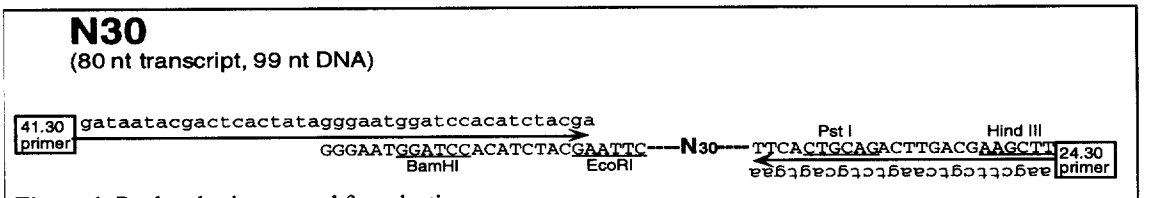

Figure 1. Pool and primers used for selection.

Characteization of clones: After 11 rounds of selection and amplification, the pool was cloned (TA cloning kit, Invitrogen, Carlsbad, CA) and sequenced using standard dideoxy methods. Individual clones were characterized using a nitrocellulose filtration assay similar to that employed for selection.

Results and Discussion

Preparation of a phosphorothiolated pool: It has been shown that phosphorothioate nucleotide triphosphates can be readily incorporated into nucleic acids.[9] Therefore, we used a double-stranded DNA pool that contained 30 random sequence positions (N30) as a starting point for the synthesis of a phosphorothiolated RNA pool. This pool had previously been used to select aptamers that could bind protein targets, and a pool with a similar number of random sequence positions had been used to identify anti-bFGF aptamers.[10]

The yield of phosphorothiolated RNA following in vitro transcription and purification, though lower than that obtained with ribonucleotidetides, was nonetheless more than adequate for selection experiments. The original PSRNA pool contained approximately 5×10^{12} different sequences.

Selection of phosphorothiolated aptamers: The pool was mixed with bFGF and protein-binding species were collected by filtration, eluted, amplified, and re-selected. As the selection progressed, several steps were

Round	PCR cycles	Ratio PSRNA/bFGF	[PSRNA] nM	background	% binding	Prefiltration dilution	1 M NaCl wash	Rxn vol. μL
1	8	1.25	360					200
2	9	1.25	375					200
3	9	10	375					200
4	17	5	375					200
5	12	10	750	0.1	9.6			200
6	21	5	375			1 mL		200
7	9	7.5	560			1.5 mL		200
8	9	1	80	0.5	11.6	3 mL		400
9	9	15	1200				x	200
10	14	22.5	1800				x	190
11	10	8	640	0.9	14		x	100

Figure 2. Selection condtions for PSRNA-bFGF.

taken to progressively increase the stringency of the selection with the goal of narrowing the starting pool to one enriched with high-affinity bFGF binding species. The ratio of protein to PSRNA was generally increased throughout the selection in an effort to increase competition among binding species. After round 5, the reaction mixture was diluted with binding buffer and then filtered, in an effort to retain binding species with slow dissociation constants. After Round 8, the filter bound protein complexes were washed with a 1 M salt solution in an effort to reduce nonspecific binding. The progress of the selection was monitored after the 5th, 8th, and 11th rounds by radiolabelling the pool and assaying for binding to varying concentrations of bFGF. As can be seen in Figure 3, the amount of PSRNA bound at low protein concentrations steadily increased.

Round	Ratio of Protein to RNA			
	1:1	8:1	20:1	no protein
0	2.5	29	87	1.1
5	9.6	ND	92	0.3
8	11.6	34	ND	0.5
11	14	68	ND	0.9

Figure 3. Progress of Selection. Pools were assayed for fraction of the population that bound to bFGF after several rounds of selection. Assay conditions: 50 μL rxn volume; [PSRNA] = 75 nM; 1 h incubation in PBS @ room temperature.

After round 11 individual aptamers were cloned and sequenced (Figure 4). Examination of the sequences revealed that 9 of the 21 sequences contained a 13 nucleotide region, which could fold into a concensus stem-loop motif. Although there is some degree of variation within the loop, the residues that make up the stem, the residues closest to the stem, and the size of the loop are invariant leading us to believe that this motif could be a common structural element that imparts ability to bind to bFGF.

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ps11-8    - - ACC- - - - AGGCCAGAGCTGCCTCTAAATGCTC- - CC
ps11-13   - - - CC- ACAGGGCTTGACC- GCCTTTAAGTG
ps11-3    - - - - C- - - - AGGCTGCGGCTGCCTACAAACGCCTAATG
ps11-34   - - - - - - - - CCGGCTTGACCTGCCATCTTAAACGGTT- - CC
ps11-20   - AATCCCAATGGCTTGAACTGCCAACGAACG
ps11-10   - G- CTAAACTGGCTTGAACTGCCTTCGAACG
ps11-37   - - - - CTGACTGGCTTGGACTGCCACTAAATGTCT
ps11-6    - - - - - TG- - - GGCTTTGACTGCCACTAAATGTCTACCC
ps11-17   TAG- - - - - - - GGCTTGGGCTGCCTTAATGCCTACAA
ps11-11   CGGAGTAGCCTGCTCCTTATAATAGGCACC
ps11-15   CGGGACCTGATGAGCCCTATAACCATCATC
ps11-18   CCAGCCTGCCCTGCTATAATGGGCCTTCTT
ps11-2    GGGTACAGCAACCCTACCTAATTGCCTTG
ps11-22   CCCTGTGGGGATGCTGCAGGCCCCTATATG
ps11-24   CTCGTGTTCAGTGGCACGTAACATC
ps11-27   CTTTGGAGGTATGACGTACCTTTAAACG
ps11-32   AGCTGCCTGCTGTCCCAATGTTGGCGATAA
ps11-36   GGCGTGCCGAGCCGCTTCTTCGGCTACCTC
ps11-38   CATCTTCGGCGTTGGCCAACACTTGTACA
ps11-4    GCCCGGCGGGGTACTATGTACGCACTAAC
ps11-9    GGGCTAGCGACCTACCTTAACGCCTT
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Figure 4a. Sequences of the random regions of the DNA templates of individual clones.

Figure 4b. Structure of the 13 nt consensus motif found in the nine member family of sequences.

Specificity of phosphorothioalated aptamers: While it was clear that selection experiments with phosphorothiolated RNA molecules could return specific sequences and structures, similar to results observed with natural RNA molecules, it was unclear whether the phosphorothiolated aptamers would bind specifically to their target. Previous results with phosphorothiolated antisense oligodeoxynucleotides had revealed that phosphorothiolated nucleic acids frequently bound less specifically than their natural counterparts, and could in fact bind to non-nucleic acid targets.[11,12] Therefore, we first assayed a phosphorothiolated aptamer from the consensus family, ps11-20, for its ability to tightly bind to bFGF (Figure 5). The calculated K_d of the aptamer from its cognate protein is 1.8 ± 0.8 nM, a value that is similar to the dissociation constants of the all RNA or partially modified anti-bFGF aptamers. An anti-bFGF aptamer selected from a RNA pool exhibited a biphasic binding curve; the calculated K_d values for two interconverting conformers were 0.19 and 49 nM. An anti-bFGF aptamer selected from a RNA pool containing 2′ amino pyrimidines formed a complex with bFGF whose K_d value was 0.35 nM.[4,10] We then examined whether the

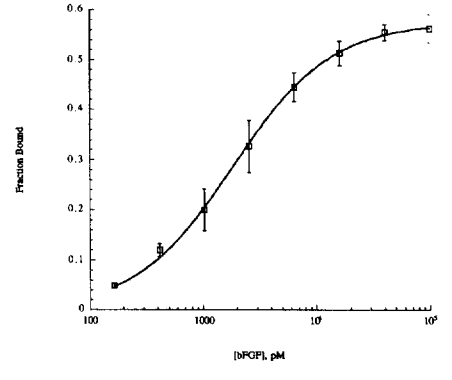

Figure 5. Binding curve of 11ps-20 with bFGF. 5 fmol radiolabelled aptamer was incubated with varying amounts of bFGF for 1 h in 100 μL PBS. The value for the dissociation constant was calculated using Kaleidograph software (Synergy Software, Reading, PA).

phosphorothiolated aptamer could bind to other targets. As seen in Figure 6, the aptamer binds not only to bFGF but also to a related growth factor, acid fibroblast growth factor (aFGF). However, the aptamer does not bind to an unrelated protein that has previously elicited aptamers, thrombin. In addition, aptamer ps11-20 and other selected clones from the consensus family were assayed for binding to other unrelated proteins, antithrombin III and VEGF, but no appreciable binding was observed (data not shown).

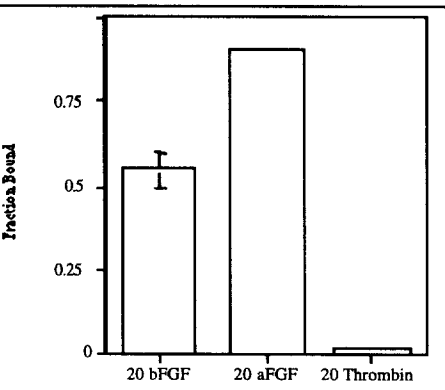

Figure 6. Specificity profile of aptamer ps11-20 with bFGF, aFGF, and thrombin. Assay conditions: [ps11-20] = 75 nM, [protein] = 300 nM in PBS, 1 h incubation.

The finding that the anti-bFGF aptamer could bind related but not unrelated targets suggested that it recognized similar sites on these targets. Previously, anti-bFGF aptamers selected from RNA or modified RNA pools had been shown to interact with heparin-binding sites on target proteins.[4,10] In order to test whether this was also the case for the phosphorothiolated aptamers, ps11-20 was assayed for binding to bFGF in the presence of increasing concentrations of heparin. Consistent with previous results, heparin effectively competed with the aptamer for binding (data not shown). These results are especially significant given that the other noncognate proteins assayed for binding, thrombin, antithrombin III, and VEGF, also contain heparin-binding sites. Thus, the aptamer may be able to identify related heparin binding sites and discriminate against nonrelated heparin binding sites; the basis for this discrimination may be the aptamer's mimicry of one of several different natural sulfated oligosaccharides of which heparin is a generic example. To further assess this possibility, the binding of selected PSRNA aptamers to other members of the FGF family is currently being investigated. If the aptamer indeed proves to be 'semi-specific' for FGF family members, it may prove to be useful as a 'shape probe' for the identification of related heparin binding sites, or as a therapeutic nucleic acid for the general inhibition of cell growth.

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