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DNA Aptamers that Bind to Chitin

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Abstract—We have succeeded in the acquisition of DNA aptamers that recognize chitin using in vitro selection. The obtained DNA aptamers have the stem-loop or bulge loop structures with guanine rich loop clusters and the clockwise B-form stems.

Introduction

Oligosaccharide antigens play essential biological roles in cellular adhesion, molecular recognition, and so on. However, little is known about the interaction concerning oligosaccharide from the viewpoints of its molecular basis. The ability to discriminate the presence of different saccharide types with relatively subtle structural differences would be of benefit in both experimental applications and diagnostics. High-affinity nucleic acid ligands, termed 'aptamers', can be selected from a random pool of nucleic acid sequences.[1–3] The isolation methodology of the ligands is one of combinatorial chemistry techniques, termed 'in vitro selection' or 'systematic evolution of ligands by exponential enrichment (SELEX)'. Such selection produces high-affinity ligands that can be propagated indefinitely with little risk of losing the stock in significantly lower cost than antibodies, and this method completely obviates the use of animals. In addition to possible ethical considerations, this also provides an opportunity to generate ligands to antigens that are toxic to humans and animals. This selection procedure involves the iterative isolation of ligands out of the random sequence pool with affinity for a defined target molecule together with PCR-based amplification of the selected oligonucleotides after each round of isolation. Using the above methodology, various RNA ligands specific to various target molecules such as amino acids, bases, nucleotides and enzymatic cofactors have been identified.[4,5] Recently, single-strand(ss)DNA ligands that bind thrombin, organic dye, ATP have been isolated via SELEX screenings.[3,6]

In the present study, DNA ligands that selectively bind to 'chitin', poly-beta-1,4-*N*-acetylglucosamine, was isolated using SELEX.

Results and Discussions

In vitro selection of the DNA oligomers specific for chitin was performed according to the procedures described in notes.[7] For the initial selection cycle, synthetic 103-mer oligonucleotides with a random region of 59 nucleotides was amplified using the forward primer (P1) and the reverse primer (P2). After 8 rounds of selections, 20 clones were sequenced[8] from the pool of selected DNAs to give seven unique sequences. Several sequences among the 20 clones were identical, suggesting multiple identification of the same clone. The obtained sequences are shown in Table 1. The length of sequence randomized in the original library was 59 nucleotides, however the length of the obtained clone's inserts varied from 54 to 61 nucleotides, because of insertions and deletions during PCR amplifications. Examination of the sequences reveals that five clones had a tendency toward G-richness containing more than 30% G-residues. All five clones, Chi No. 1, Chi No. 23, Chi No. 28, Chi No. 46 and Chi No. 52 contained G-cluster motifs that were sandwiched with palindrome sequences.

The predicted secondary structures of the aptamers were illustrated in Figure 1, in which the regions around palindrome sequences of each aptamers were indicated. All of these represented typical stem-loop or bulge-loop structures. Percentages of G-residues of both the entire random regions and the individual loops of the aptamers

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Table 1. Sequences of chitin-binding DNA aptamers

Aptamers	Sequences[a]
Chi No. 1 (61)[b]	CCCAGCAGCACTGGTAGTGAGGCAGTTCACCGGTGGGGCGGTGAGTTTGGCTGCTATTTAT
Chi No. 23 (54)	TGCGCATGTGAAAGGTTGCCTAACTGGACAGGGTTTAGGAGCGACTAGACACAG
Chi No. 28 (59)	GGCAAGATGTGCCCAGAACAGTTGCTTGTATGGTGGGGAGCGTCCATATTGGCTTAAAC
Chi No. 35 (52)	ACCTGCGATAGGTAGTTTGTTTCTGTTGCCCTAGCGTCCGTCGTAACAAGTCA
Chi No. 46 (61)	CCGTAACCCTGCGGGGGGGGGAGAAGGCAATGGGGGACAACTCGCCGGTAGCCATCCATAT
Chi No. 52 (61)	CCTAAGGGGGGACTCAGCATTTTGTGCGGGCGGCGCTAACACAATCAGATAGAGCGGGGTT
Chi No. 57 (60)	CGTACAGTGGGCTATATTGCTGACGTAAGAGCTGCTTTCAATGCATTGGCATAGATGTCA

[a]The random sequences between the priming sites are shown.
[b]Numbers in parenthesis indicated the length of N59-random regions.

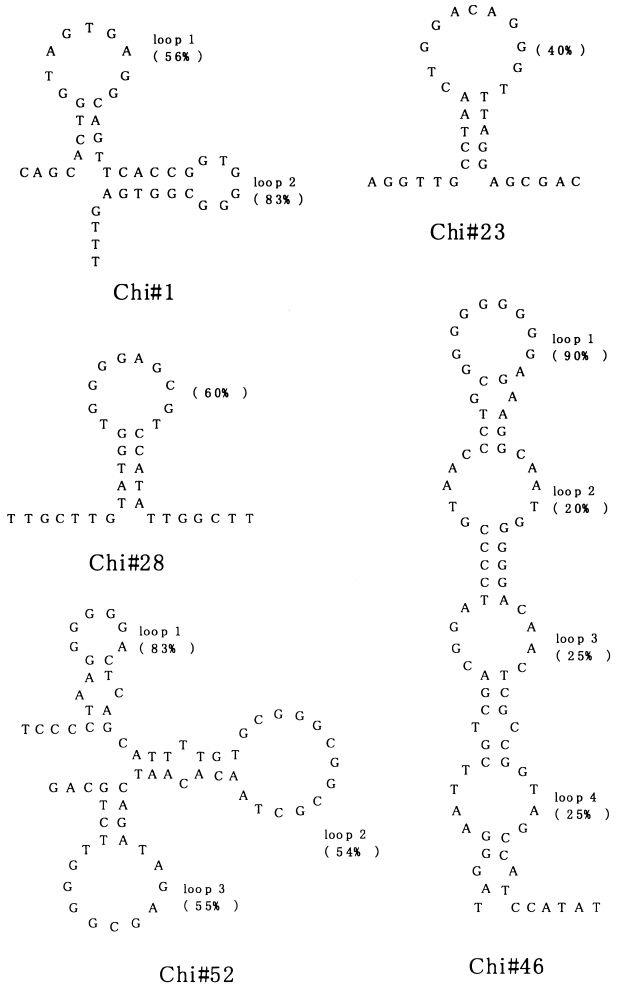

Figure 1. Possible secondary structures of chitin aptamers. Numbers in parentheses indicate guanine-contents (percentage) in each loop.

were also shown in Figure 1. The percentages of guanine bases contained in the whole random region (N59 region) are moderately high (approximately ca. 30%). However, the guanine contents in most of the loops show high G-richness (more than 50%). Therefore, the stem-loop or the bulge-loop, having the guanine-cluster in the inner loops, might be required for the recognition of chitin, though its detail mechanism is not clear.

The aptamers specific to cellulose reported by Yang et al. are also guanine-rich and are constructed by guanine-clusters with thymine spacers.[9] These suggest that the guanine cluster would be necessary for recognition of saccharide. The above mentioned cellulose aptamer sequences showed the possibility of forming guanine-cluster but any other secondary structures. However, the obtained chitin-aptamers in this study represented typical stem-loop or bulge-loop structures. Furthermore, the stems are constructed by four to five base pairs in all the stem-loops and judging from the fact that purine bases and pyrimidine bases do not alternately line up, those would have the structure of clockwise B form. Both Chi No. 1 and Chi No. 52 have the plural number of stem loops in which each loop is connected in the form of hinges. These structural features would be important for exclusive recognition of 'chitin'.

The obtained five clones were subjected to chitin binding assay[10] and the results are listed in Table 2. All aptamers except Chi No. 1 revealed the binding capability to chitin-beads. Especially Chi No. 46 and Chi No. 52 strongly bound to chitin-bead in 52 and 73% of binding efficiencies respectively, nevertheless the original library showed very little affinity below 1%. Chi No. 23 and Chi No. 28 showed moderate binding to chitin-beads, however, Chi No. 1 was observed not to bind to the target. As an evaluation of the specificity of the aptamer interactions with chitin, the same assay was used except that cellulose was one of the similar polysaccharides tested as the target molecule (Table 2). In the results, selected aptamers represented around 45% of the binding efficiencies to cellulose. However, the original library also showed a moderate binding efficiency to cellulose (20%), which was a different tendency from the chitin-bead assay. These findings show that the original library's high affinity to cellulose can attribute to primer regions of the library pool. Anyway,

Table 2. Binding assay of DNA aptamers Chi No. 46 and Chi No. 52

Target	Chi No. 1	Chi No. 23	Chi No. 28	Chi No. 46	Chi No. 52	Original library
Chitin-bead	< 1%	18%	6%	52%	73%	< 1%
Cellulose	46%	47%	43%	NT[a]	45%	20%

[a]NT means 'not determined'.

the binding efficiency of Chi No. 52 to cellulose was apparently less than the value for chitin-beads (Table 2). These results could indicate that aptamer Chi No. 52 selectively bound to chitin-beads.

In conclusion, the present study demonstrated that the in vitro selection method is applicable to obtain the DNA aptamer that selectively recognizes polysaccharide, which physiologically has an important meaning.

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7. In vitro selection deoxyribonucleoside triphosphate (dNTP) was obtained from Pharmacia Biotech. Co. (Sweden). AmpliTaq DNA polymerase was purchased from Perkin–Elmer Co. (USA). Custom synthesis of DNA was performed by Sawady Technology Co. (Japan). For the initial selection cycle, synthetic 103-mer oligonucleotides with a random region of 59 nucleotides, 5′-TAGGGAATTCGTCGACGGATCC-N59-CTCCAGGTCGACGC-ATGCGCCG-3′, was amplified using the primer, 5′-TAATACGACTCACTATAGGGAATTCGT CGACGGAT-3′ (P1) and 3′-GTCCAGCTGCGTACGC GGC-5′ (P2). The synthetic ssDNA (5 μg) was amplified using 12 polymerase chain reaction (PCR) cycles (one cycle: 94 °C, 15s; 55 °C, 15s; 72 °C, 15s) in 100 μL of PCR solution (25 units mL^{-1} of AmpliTaq DNA polymerase, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.02 mM dNTPs, primer P1 (0.5 μg) and primer P2 (0.5 μg)). The amplified double-stranded (ds) DNA were purified using 3% low-melting agarose gel (NuSieve GTG, FMC BioProducts (Rockland, ME, USA) in order to remove unreacted primers and dNTPs. ssDNA was then obtained from the dsDNA by 45 additional PCR cycles using only the P1 primer (asymmetric PCR). The PCR-ssDNA was purified using NuSieve GTG agarose gel and precipitated with ethanol. Two hundred microliters of the ssDNA pool (3 μg) in a binding buffer (100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–acetate (pH 8.0)) was heated at 95 °C, and allowed to cool down to room temperature. The DNA library was loaded onto the chitin-bead (NewEngland Bio Labs, USA) column (bed volume 0.5 mL) prewashed with 20 bed volume of binding buffer. After 30 min of incubation at room temperature, the column was rinsed with 5 mL of binding buffer. The bound DNA was eluted with 3 mL of distilled water. The eluted ssDNA solution was added 1/200 volumes of glycogen (Behringer Mannheim) and precipitated with ethanol, rinsed with 70% of ethanol and dissolved in 30 mL of distilled water. The recovered ssDNA was amplified by PCR and subjected to the next selection cycle. This process was repeated 8 times.
8. Obtaining monoclonal aptamers and DNA sequencing. Double stranded DNA fragments were prepared from ssDNA pools after 8 rounds of selection, and ligated into pCR2.1 (invitogen). Transformation into *Escherichia coli* DH5a was followed by direct PCR screening of plasmid-containing bacterial colonies. One hundred microliters of the colony PCR solution consisted of: 25 units mL^{-1} of AmpliTaq DNA polymerase, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.02 mM dNTPs, M13 forward primer (50 mM), M13 reverse primer (50 mM) and a small amount of bacterial cells. The cycling protocol was 30 cycles at 94 °C for 1 min, 46 °C for 1 min and 72 °C for 2 min. Plasmid DNA was isolated by the alkaline-SDS method. DNA sequencing was performed using ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin–Elmer, USA). The secondary structure of the ssDNA aptamer was predicted using GENETIX MAC.
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10. Chitin binding assay. α-^{32}P-dGTP was incorporated into single-stranded DNA by using asymmetric PCR and isolated after NuSieve GTG agarose gel electrophoretic separation. ^{32}P-labeled ssDNA fragments were subjected to chitin bead followed by the washing with binding buffer according to the same procedures described above. The amount of both loaded ssDNA fragments and unbound fractions were monitored by Cerenkov counting. The ability of the ssDNA fragments to bind to the chitin bead was determined by comparing the amount of DNA loaded onto the column with that eluted from the column by the binding buffer.