Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2927–2930

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SELEX for Tubulin Affords Specific T-Rich DNA Aptamers

Ei-ichiro Fukusaki,[a,*] Tomohisa Hasunuma,[a] Shin-ichiro Kajiyama,[a] Atsushi Okazawa,[a] Tomohiko J. Itoh[b] and Akio Kobayashi[a]

[a]*Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1, Yamadaoka, Suita 565-0871, Japan*
[b]*Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan*

Received 25 May 2001; accepted 24 August 2001

Abstract—We succeeded in acquiring two DNA aptamers that selectively recognize tubulin by the SELEX method. A pool of single-stranded oligo-DNAs including a random region of 59 nucleotides was screened by SELEX for tubulin purified from calf-brain as a target. After 20 repetitions of selection round, the library converged on specific T-rich sequences. The binding activity of T-rich clones was analyzed by the SPR sensor to determine their dissociation constants to be in the order of 10 μM. ©2000 Elsevier Science Ltd. All rights reserved.

In vitro selection developed into a powerful tool for screening of a randomized oligonucleotide pool to acquire DNA or RNA molecules called 'aptamer' that specifically bind to target molecules. This new technique is called 'systematic evolution of ligands by exponential enrichment (SELEX)' method.[1,2] This method, using a randomized oligonucleotide library as a screening source, enables the selected molecules to be reamplified by the PCR method. Therefore, repetition of the affinity screening can yield a desired aptamer effectively with low cost. Using this method, a number of aptamers that specifically recognize many kinds of targets, such as organic compounds, oligonucleic acids, peptides, and proteins have been obtained.[3,4] The acquisition of ligands for targets that are not suitable for the conventional immunological techniques is possible by the in vitro SELEX method. Moreover, screening under conditions that are remarkably different from physiological conditions can be carried out.

In this study, we present an acquisition of DNA aptamers that bind to tubulin using SELEX. Tubulin is the main component of microtubules, which are involved in a variety of biological events such as intracellular transport, cellular motility, and construction of cytoskeletons.[5] These cytoskeletal elements also act pivotal roles in mitotic spindle assembly and cell division. Numerous ligands that bind to tubulin and affect its assembly have been employed as anticancer drug candidates.[6] Tubulin ligands have been also believed as useful probes for studying the structures of tubulin molecules.

Results and Discussion

Microtubule proteins prepared from crude calf-brain extract by repetitive polymerization and depolymerization were applied to ion exchange chromatography to yield pure tubulin according to the reported procedure.[7] Purified tubulin (50 nM) dissolved in PM buffer (100 mM PIPES pH 6.9, 1 mM EGTA, 0.5 mM $MgSO_4$; 100 μL) was loaded into a well of a high protein binding micro titer plate (Corning Inc., Corning, NY, USA), and incubated overnight at 4 °C. After excess tubulin was removed and the well was washed three times with PBS plus 0.1% (v/v) Tween-20, the well was blocked for 30 min by Block Ace™ (Snow Brand Milk Products Co., Tokyo, Japan), followed by three washes with PBS plus 0.1% (v/v) Tween-20 and selection buffer (100 mM PIPES pH 6.9, 1 mM EGTA, 5 mM $MgSO_4$, 100 mM NaCl). Screening of the random DNA pool to acquire specific DNA ligands to tubulin was performed according to the method reported[8] with minor revisions. For the initial selection, synthetic 103-mer oligonucleotides with a random region of 59 nucleotides, 5′-TAGGGAATTCGTCGTCGTCGACGGATCC-N59-CTGCAGGTCGACGCATGCGCCG-3′, were amplified over 12 cycles of PCR (94 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s) using the forward primer, 5′-TAATACGACTCACTA-

*Corresponding author. Tel.: +81-6-6879-7424; fax: +81-6-6879-7424; e-mail: fukasaki@bio.eng.osaka-u.ac.jp

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00584-4

TAGGGAATTCGTCGAC-3′ (P1) and the reverse primer, 5′-CGGCGCATGCGTCGACCTG-3′ (P2). The random ssDNA pool was obtained from the double-stranded DNAs (dsDNAs) by an additional 45 cycles of asymmetric PCR using only the P1 primer. The PCR products were purified by 8% polyacrylamide gel electrophoresis. After preselection on a Block Ace™ immobilized plate, the ssDNA pool (5–10 μg) in the selection buffer was subjected to the tubulin immobilized plate (100 μL) and incubated at 4 °C for 30 min. Unbound ssDNAs were washed away by the selection buffer (300 μL, three times). Bound ssDNAs were eluted with the anti-tubulin antibody (Transformation Research Inc., Framingham, MA, USA) in PBS (100 μL). The PBS with which the well was washed three times was combined with the eluted solution. From the combined solution, DNA was precipitated by ethanol after deproteinization with TE-saturated phenol. The recovered ssDNAs were amplified with PCR (12–16 cycles) and the obtained dsDNA pool was subjected to asymmetric PCR as described above to yield the ssDNA pool for the next selection. Making the process from the preparation of the ssDNA pool to the recovery of the bound ssDNA population to be one round, the selection was repeated 20 rounds.

After selection, the specific ssDNA pool was subjected to a tubulin binding assay using an SPR sensor (SPR670 biosensor, Nippon Laser & Electronics Lab., Nagoya, Japan) (Fig. 1). The SPR sensor is a useful instrument for real time observation of molecular recognition and kinetic studies based on the principle of surface plasmon resonance. Streptavidin was immobilized on a dithiodibutyric acid-coated sensor chip after activation with water-soluble carbodiimide (16 mM) and *N*-hydroxysuccinimide (13 mM). A 10 μL/min continuous flow of the selection buffer was applied at 20 °C. After blocking with Block Ace™, the 5′ biotinylated ssDNA pool was immobilized onto the surface of the sensor chip. Tubulin was then applied to evaluate its interaction with the immobilized ssDNAs. Subsequently, the surface of the sensor chip was regenerated with 0.03% SDS. Next BSA solution was applied for the negative control measurement in an equal concentration to that of the tubulin solution. In the series of the experiment, 5′-biotinilated original library ssDNA pool was employed as a negative control. As a result, it was shown that tubulin was bound to the ssDNA pool after 20 rounds of selection more firmly in comparison with the original ssDNA pool before selection (Fig. 1). In contrast, there was hardly any observable difference in adsorption before and after selection when BSA was run. So, it is revealed that the selected DNA pool binds specifically to tubulin. These results indicated that enrichment of a population that binds specifically to tubulin from the randomized ssDNA pool by SELEX was achieved.

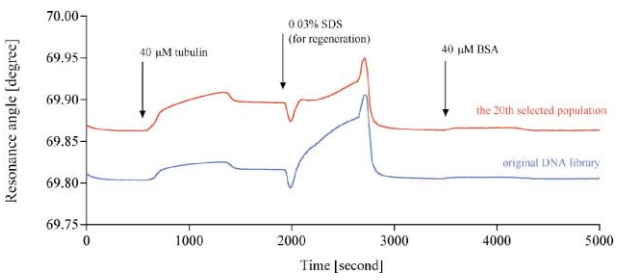

Figure 1. SPR analysis of aptamer specificity. 40 μM tubulin and control protein (bovine serum albumin) were injected into flow cells where biotinylated either the 20th selected population (red line) or orginal library (blue line) was immobilized on a streptavidin coating sensorchip. 0.03% SDS was injected for regeneration of the sensorchip surface. The arrows indicate the starting points of injection.

DNA sequences in the selected DNA pool were analyzed as shown bellow. The PCR products obtained after 20th round of selection were subcloned into pUC18, then introduced into *Escherichia coli* (DH5a). Twenty-one clones were randomly picked for DNA sequencing analysis. Plasmid DNAs were isolated by the alkaline lysis method and their sequences were determined by the dye terminator method using ABI PRISM 310 Genetic Analyzer (Perkin–Elmer Applied Biosystems Inc., Foster City, CA, USA). Eighteen clones shown a specific bias, thymine repeat or guanine repeat. These clones were classified into three categories, 'T-rich', 'T/G-rich', and 'G-rich' families (Fig. 2). Among them, several clones significantly converged into 'T-rich' family. Previous report concerning SELEX screening only involved DNA aptamers representing 'G-quartet',[9–11] 'G-wire'[12] or 'G/T-rich'[13] sequences. This result is the first report of 'T-rich' aptamers. It is known that the T-rich part exists in the centromere of budding yeast *Saccharomyces cerevisiae*.[14] The existence of interaction between tubulin and centromere might be indicated from present results. In addition, it is known that there is an enormous repeating region called 'alphoid DNA' consisting of 171 bp unit in the primary stenosis region of the chromosome in mammalian. Microtubule seems to adhere to this region.[15] However a homology search study of the clones obtained in this study by BLAST and FASTA on network service (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html) showed no homology sequence in any organism.

Among the 21 clones, four clones converged into the same specific sequence named 'tuda-1' and other three clones converged another sequence named 'tuda-2' (Fig. 2). Further analysis of these two sequences was performed. Prediction of secondary structure of tuda-1 and tuda-2 by the computer algorithm GENTIX-MAC indicates that these T-rich regions should form stem-loop structure (Fig. 3). The interaction between tubulin and these aptamers was evaluated by SPR sensor technology. Both tuda-1 and tuda-2 showed specific binding to tubulin in a dose-dependent manner (Fig. 4). A kinetic study based on the amount of sensorgram signal change revealed that tuda-1 and tuda-2 had apparent dissociation constant of 45.0 and 19.4 μM, respectively (Table 1). A control experiment, in which the original library was used instead of tuda-1 or tuda-2, showed no signal change when tubulin was run suggesting the original library does not bind tubulin specifically.

In this study, we succeeded in acquiring two DNA aptamers that selectively recognize tubulin by the

			%A	%T	%G	%C
	T-rich family					
(4)	tuda-1:	ATGCTCGCGCCTGCTGTGTTGTTGCTTGGTTTGGTCTTTTTTTGGTTTGTTTTGGGTT	2	53	31	24
(3)	tuda-2:	GTTGGGCTACAGTACTTCCCCTTTTTTCTCTTCCTCCTTCCTATCGCTCTCC	8	42	12	38
(1)	tuda-3:	AGGCTATTCGTTTTGTGTTTCTTTTCCTGGTTTTGTTATTTTTTGTTGTTGTTTTTTGG	5	64	22	8
(1)	tuda-4:	GAATTCGTTTGTGTGCGGAGGTGGTTGTTTGTTTTTTTGTTTCTTTGTTTTGTTTG	5	59	30	5
	T/G-rich family					
(1)	tuda-5:	AGATATGGTTTTGTTGTGCGTTATGTTGTGTTTTTGGGGTCTCTTTTTGGGTGTTTTGT	7	56	32	5
(1)	tuda-6:	TTAAGTTTGCTGTTAATTGGTGCGTATCTCTGTAGTAGAGTCAGTTAGCGGTTTTGTGGT	17	43	30	10
(1)	tuda-7:	ACGTTCCGCTGGTATAGTGAGTGACCTTGGTCTGGTGGATGCGTTTATTCGTGGTCCTC	12	36	32	20
(1)	tuda-8:	CTTAGTGATATCAGGTGTCGTTACTTCAGGGTGAGTTCATGTGTTGCTTAAAGTTATTG	20	41	27	12
	G-rich family					
(1)	tuda-9:	TTGGTGGGTTGTAGGCAGGCGTGGGCACTGTTTGAGAAGGACGTGTTTGGTCTAT	15	31	42	11
(1)	tuda-10:	ATGTATTACTAGGTCGTTGACGGAGAAGGGGGGTAGGTGCTTGGGTGTGTCTGTTTGGT	15	34	42	8
(1)	tuda-11:	TAGCTTGGTTGGCTTGGTGCCGTAGCTGTCAGGTAGGTCTGGGAATCGCTGGGTGAA	14	30	40	16
(1)	tuda-12:	TGGTGGGATGTCGTTCGAAGATTGTTTGCGTGTGTGCCGTAGATATGCTGGGGTTGCAT	14	36	39	12
(1)	tuda-13:	CGAATGGGGAGTGGGACCGAATCTTGGTGTGTGTTCTGAAGCAGGGGTTCAATATGGTT	20	29	39	12
	Other sequences					
(1)	tuda-14:	TCAAGATCGGTTGCCGTGGGTTACAGTGGCGATTGTTACAAGGTGCAATAGTCCCTCGC	20	27	31	22
(1)	tuda-15:	ATCGAAAGGTGGGTCGGATTAGTATGAGAGGTCTTCGCATTCGGTGCTGGGATCCTTTA	20	31	34	15
(1)	tuda-16:	CGAGGCTTAAGGCGCAACTAATCTCGTTGGTGGTTCGCCACAGGTGTTCCAAC	21	25	28	26

Figure 2. Sequences in random region of selected DNAs. Based on the ratio of repeated bases, 18 clones were classified into three categories. On the left side, the number of clones with identical sequences are shown in parentheses and, on the right, the frequency of occurrence for each base is shown.

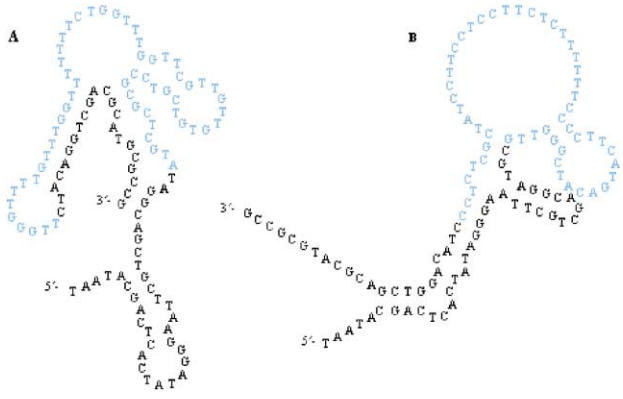

Figure 3. Possible secondary structures of (A) tuda-1 and (B) tuda-2. The sequences in random region are shown in blue letters.

SELEX method. The aptamers converged to a previously unexampled T-rich sequence. We have carried out SELEX screening for the other targets using random oligo-DNA library that is completely identical with the one used in this study and we have succeeded in the acquisition of various other aptamers. The aptamers obtained by SELEX for a neutral polysaccharide chitin converged to stem-loop structures with G-rich loops.[16] They also converged to G-rich sequence when the aromatic compound hematoporphyrin was made as a target. However, the SELEX screening for hematoporphyrin yielded not the stem–loop structure but the guanine quadruplex wire structure.[17] It is very interesting that an identical library should converge into completely different sequences by SELEX screening for the different targets. Our results suggested that SELEX

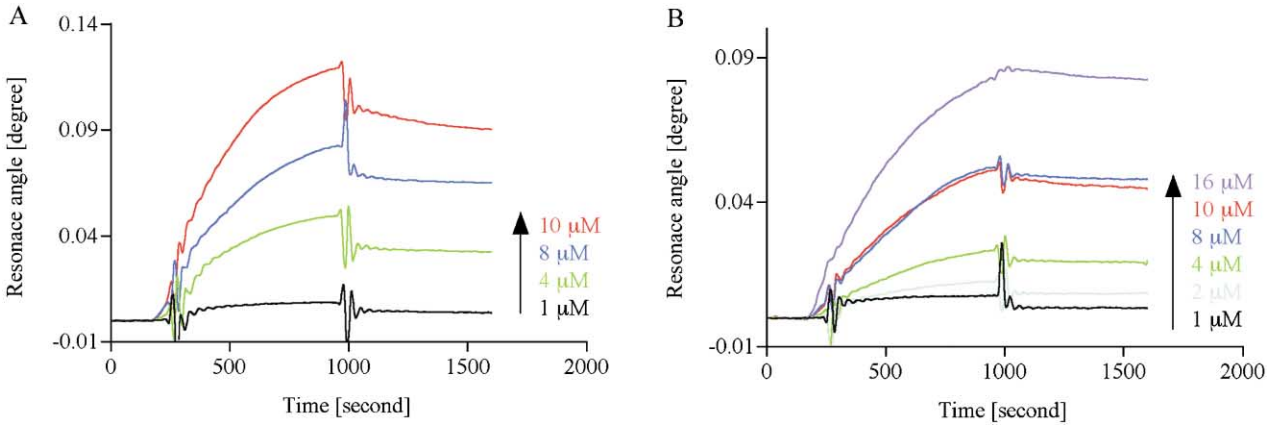

Figure 4. Overlay plots of the interactions between DNA aptamers and tubulin. Increasing concentration of tubulin as indicated by the arrow were injected into flow cells where biotinylated either (A) tuda-1 or (B) tuda-2 was immobilized on a streptoavidin coating sensorchip. Signals from only a streptoavidin coating channel served as baseline and were subtracted to the signal change observed when tubulin interacted with immobilized ssDNAs. Conditions of SPR analysis is same as that described in Figure 1.

Table 1. Kinetic constants for tubulin binding DNAs. The rate constants, k_d and k_a were determined from sonsorgram using kinetic evaluation software. K_D was calculated as k_d/k_a

DNA	K_D (M)	k_d (s^{-1})	k_a ($M^{-1}s^{-1}$)
Tuda-1	4.50×10^{-5}	3.44×10^{-3}	7.65×10
Tuda-2	1.94×10^{-5}	1.38×10^{-3}	7.10×10
Original library	N.D.[a]	N.D.	N.D.

[a]N.D., not detected.

screening could be a powerful tool to obtain specific aptamers for various targets, such as sugars, proteins and aromatic compounds with identical libraries. The data accumulated by systematic SELEX screening of identical libraries for different targets should be useful for the practical prediction of converging sequences for various targets.

Acknowledgements

This research was supported in part by a grant-in-aid (No. 12019242) for Science Research from the Ministry of Education Science, Sports and Culture, Japan.

References and Notes

1. Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818.
2. Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.
3. Famulok, M.; Jenne, A. *Curr. Opin. Chem. Biol.* **1998**, *2*, 320.
4. Famulok, M. *Curr. Opin. Chem. Biol.* **1999**, *9*, 324.
5. Bryan, J. *Fed. Proc.* **1974**, *33*, 152.
6. Dumontet, C.; Sikic, B. I. *J. Clin. Oncol.* **1999**, *17*, 1061.
7. Murata, M.; Ito, T. J.; Kagiwada, S.; Hishida, R.; Hotani, H.; Ohnishi, S. *Biol. Cell* **1992**, *75*, 127.
8. Conrad, R. C.; Giver, L.; Tian, Y.; Ellington, A. D. *Methods Enzymol.* **1996**, *267*, 336.
9. Padmanabhan, K.; Padmanabhan, P. K.; Ferrara, J. D.; Sadler, J. E.; Tulinsky, A. *J. Biol. Chem.* **1993**, *268*, 17651.
10. Huizenga, D. E.; Szostak, J. W. *Biochemistry* **1995**, *34*, 656.
11. Weiss, S.; Proske, D.; Neumann, M.; Groschup, M. H.; Kretzschmar, H. A.; Famulok, M.; Winnacker, E. L. *J. Virol.* **1997**, *71*, 8790.
12. Sugimoto, N.; Toda, T.; Ohmichi, T. *Chem. Commun.* **1998**, 1533.
13. Tracy, R. B.; Baumohl, J. K.; Kowalczykowski, S. C. *Genes Dev.* **1997**, *11*, 3423.
14. Fitzgerald-Hayes, M. *Yeast* **1987**, *3*, 187.
15. Jorgensen, A. L. *Dan. Med. Bull.* **1997**, *44*, 522.
16. Fukusaki, E.; Kato, T.; Maeda, H.; Kawazoe, N.; Ito, Y.; Okazawa, A.; Kajiyama, S.; Kobayashi, A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 423.
17. Okazawa, A.; Maeda, H.; Fukusaki, E.; Katakura, Y.; Kobayashi, A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2653.