Biotechnol Lett (2010) 32:643–648
DOI 10.1007/s10529-010-0200-5

ORIGINAL RESEARCH PAPER

Screening of DNA aptamer which binds to α-synuclein

Kaori Tsukakoshi · Ryuichi Harada · Koji Sode · Kazunori Ikebukuro

Received: 22 September 2009 / Revised: 18 December 2009 / Accepted: 21 December 2009 / Published online: 29 January 2010

Abstract α-Synuclein is a native, unfolded protein that causes several neurodegenerative diseases such as dementia with Lewy bodies and Parkinson's disease. We have now identified the first DNA aptamers against α-synuclein using native PAGE applied to the SELEX method. We call this aptamer "M5-15"; it is the α-synuclein-bound aptamer and was isolated after four cycles of screening. M5-15 is composed of three stem-loop structures that may play an important role in the binding to α-synuclein. Moreover, M5-15 specifically binds to the α-synuclein monomer and oligomer. We expect that this aptamer will become a useful tool in α-synuclein analysis and diagnosis.

Keywords α-Synuclein · Alzheimer's disease · DNA aptamer · Lewy bodies · Parkinson's disease

Introduction

Aptamers are target-binding molecules that consists of DNA or RNA; it is selected from random sequence pools in vitro using a process referred to as "SELEX" (*s*ystematic *e*volution of *l*igands by *ex*ponential enrichment) (Ellington and Szostak 1990; Tuerk and Gold 1990). Aptamers can be raised against almost any type of target; small molecules, chemical compounds, proteins, and so on. Aptamers are easily and inexpensively synthesized and chemically modified. Furthermore, they have the potential of undergoing structural change when they bind to the target, and they have not only high affinity and specificity for the target, but many other interesting features which make them useful as molecular recognition elements.

A causative role for α-synuclein in dementia with Lewy bodies (DLB) and Parkinson's disease (PD) pathogenesis is supported, since the major fibrillar protein component of Lewy bodies in both sporadic and familial PD is α-synuclein. To investigate a connection between PD and α-synuclein, several antibodies have been developed as tools for the observation and analysis of α-synuclein. Recently, the α-synuclein oligomer has become a very important target for the resolution of cytotoxicity in several neurodegenerative diseases. The antibody technique against α-synuclein has been expanded to the detection of the oligomer (El-Agnaf et al. 2006; Paleologou et al. 2009). Furthermore, soluble α-synuclein oligomer was detected in the brain of post-mortem DLB patients (Paleologou et al. 2009). Therefore, the ligands which recognize and bind specifically to α-synuclein are required for the determination of α-synuclein in synucleinopathy.

K. Tsukakoshi · R. Harada · K. Sode · K. Ikebukuro (✉)
Department of Biotechnology and Life Science,
Tokyo University of Agriculture and Technology,
2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan
e-mail: ikebu@cc.tuat.ac.jp

Aptamers have been obtained against amyloid beta (Ylera et al. 2002; Takahashi et al. 2009) and a prion (Weiss et al. 1997; Proske et al. 2002; Rhie et al. 2003; Ogasawara et al. 2007), both of which cause neurodegenerative disorders. However, aptamers against α-synuclein have not yet been reported. In this study, we achieved the isolation, by SELEX method using native PAGE, of novel aptamers which binds to α-synuclein. We have been screening for aptamers against several targets and trying to apply those aptamers to the creation of novel biosensing systems. In fact, we have succeeded in obtaining new aptamers and developing original sensors in previous studies (Yoshida et al. 2006, 2008). These aptamers can be applied to biosensors as molecular recognition elements for the detection of α-synuclein.

Materials and methods

Preparation of recombinant α-synuclein and α-synuclein oligomers

The α-synuclein structural gene was subcloned into pET-28(a) vector (Novagen), and the protein used in this study was expressed in *Escherichia coli* BL21 (DE3), as described previously (Kobayashi et al. 2006). α-Synuclein oligomers were prepared by a method established by Ding et al. (2002). Lyophilized α-synuclein was dissolved in MQ and then flash-frozen in liquid N_2 before relyophilization. The dehydrated powder was dissolved at 1 mM in PBS, pH 7.3, and incubated for 2 h at room temperature. The insoluble aggregates were removed by 0.22 μm filter (Millipore), and the soluble samples were loaded onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with PBS and eluted at a flow of 0.5 ml/min. NaN_3 was added to the purified samples to 0.02%, and the samples were stored at 4°C. The concentration of oligomer was determined by DC protein assay kit and by measuring the density of Coomassie Brilliant Blue (CBB)-stained SDS–PAGE using BSA as a standard.

SELEX protocol using native PAGE

A FITC-labeled single-strand DNA library containing a 30-mer randomized region and 18-mer primer binding sequences at both ends was used (5′-FITC-ATA GTC CCA TCA TTC ATT-N_{30}-AGA TAT TAG CAA GTG TCA-3′). This library was amplified using a forward primer (5′-AAT GAA TGA TGG GAC TAT-3′) and a reverse primer (5′-TGA CAC TTG CTA ATA TCT-3′). These synthesized oligonucleotides were purchased from Invitrogen. The modified DNA library was heated at 95°C for 3 min and then gradually cooled to 25°C at a rate of 2°C per min in TBS buffer (10 mM Tris/HCl, 150 mM NaCl, 5 mM KCl, pH 7.4) in order to fold the structures. This library was incubated with the prepared α-synuclein monomer for 30 min at room temperature. α-Synuclein was used after ultracentrifugation (150,000 × *g*, 1 h, 4°C) to remove the aggregates. This mixture was separated on a 15% (v/v) homogeneous gel by native PAGE. Then, the DNA positions were detected by FITC fluorescence, and the protein was stained by CBB. After separation, the portion of the gel containing the α-synuclein band was clipped, and the target-bound DNA was extracted and amplified by PCR. After four rounds of screening, the oligonucleotides in the screened library were subcloned using the pGEM-T Vector System (Promega) and sequenced. The secondary structures of individual sequences at 25°C including 150 mM Na^+ were predicted by means of the Zuker DNA folding program (Mathews et al. 1999).

Gel shift assay

The FITC-labeled aptamers were heated and cooled in order to fold the structures, as described above. The aptamers were diluted to 5 μM and α-synuclein monomer at various concentrations was mixed in for 30 min at room temperature, after which it was separated on a 15% (v/v) homogeneous gel. Finally, the aptamers which were bound to α-synuclein were checked by monitoring the FITC fluorescence.

Aptamer blotting assay to investigate the specificity of M5-15 against α-synuclein and its binding to the α-synuclein monomer and oligomer

The FITC-labeled M5-15 was heated at 95°C for 30 min and then gradually cooled to 25°C at 2°C per min in Tris/HCl buffer (10 mM Tris/HCl, pH 8.0). α-Synuclein monomer, $A\beta_{1\text{-}42}$ (Peptide Institute, Inc.) and BSA (Sigma) were immobilized. A 140-pmol aliquot of each protein was spotted onto a

nitrocellulose membrane, and the membrane was blocked with 4% (v/v) skim milk. The aptamer was diluted to 5 μM with Tris/HCl buffer and incubated with these proteins. After washing, the membrane was incubated with 1,000-fold-diluted HRP-conjugated anti-FITC antibody, and the HRP activity was visualized to evaluate the affinity of the aptamer.

In the case of the evaluation of the binding of the aptamer to the α-synuclein monomer and oligomer, FITC-labeled PolyT consisting of 66 mer thymine was employed similarly to M5-15 as a control. α-Synuclein monomer and/or α-synuclein oligomer was immobilized on a nitrocellulose membrane, and the protein-immobilized membrane was blocked with 4% skim milk. The protein-immobilized membrane was incubated with M5-15 or PolyT diluted to 5 μM and then with HRP-conjugated anti-FITC antibody. The binding with each protein was detected as described above.

Results

Selection of the aptamers

Although there are several screening methods for DNA aptamers, we used the gel shift assay. Screening for the aptamer using α-synuclein immobilized on a nitrocellulose membrane was unsuccessful, possibly due to the structural change of α-synuclein upon immobilization onto the membrane (data not shown). We concluded that it is not appropriate to immobilize α-synuclein onto a support for the purpose of screening because it is a natively unfolded protein under physiological conditions (Weinreb et al. 1996). Circular dichroism (CD) analysis by Weinreb et al. (1996) has shown that the structure of α-synuclein is sensitive to the surrounding environment. In the selection process of native PAGE, owing to the incubation with α-synuclein in solution, α-synuclein may be able to retain its native structure.

Fourteen sequences were obtained from the fifth-round DNA library, and predicted the structures of each aptamer by Zuker DNA folding program. We adopted the structure with the smallest change in the Gibbs free energy as the state of the aptamer. There were no identical sequences, but most aptamers were predicted to have some stem-loop structures. The binding ability of seven oligonucleotides to α-synuclein was investigated using the gel shift assay. The binding ability of several aptamers to α-synuclein was strong compared with that of the initial DNA library, and M5-15 was identified to bind to α-synuclein with the highest affinity.

Investigation of binding by the gel shift assay

The binding of the M5-15 aptamer to α-synuclein was investigated from the viewpoint of concentration dependence. α-Synuclein and M5-15 at different concentrations were incubated for 30 min, and the binding capacity of M5-15 was analyzed by the gel shift assay (Fig. 1). The results suggest that the binding of M5-15 to α-synuclein becomes saturated at 25 μM α-synuclein.

The results of the gel shift assay, on the other hand, indicate that the obtained aptamers, including M5-15, may have two structures, one which binds to α-synuclein and one which does not. M5-15 was predicted to form three stem-loop structures (Fig. 2a), one of which is composed of a randomized region only and two others which are composed of a primer region and a randomized region. To determine which sequences play an important role in M5-15 binding, three truncated mutants of M5-15 were constructed as described below. The structures of M5-15 and the truncated mutants were predicted by M-fold program. The first truncated aptamer consisted only of a 30-mer randomized region, which includes one stem-loop

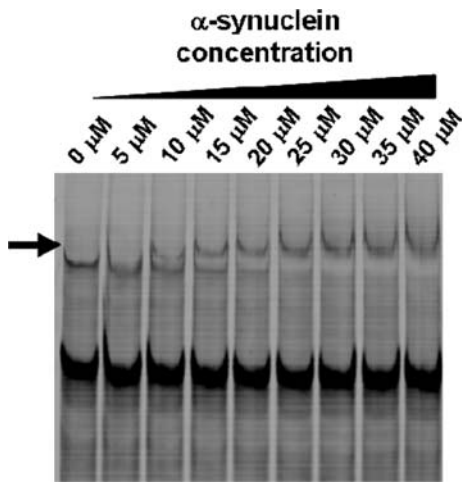

Fig. 1 Gel shift assay of M5-15 and α-synuclein. 5–35 μM α-synuclein and 5 μM M5-15 were incubated for 30 min and analyzed. FITC fluorescence-modified M5-15 was detected. The *arrow* indicates the position of α-synuclein

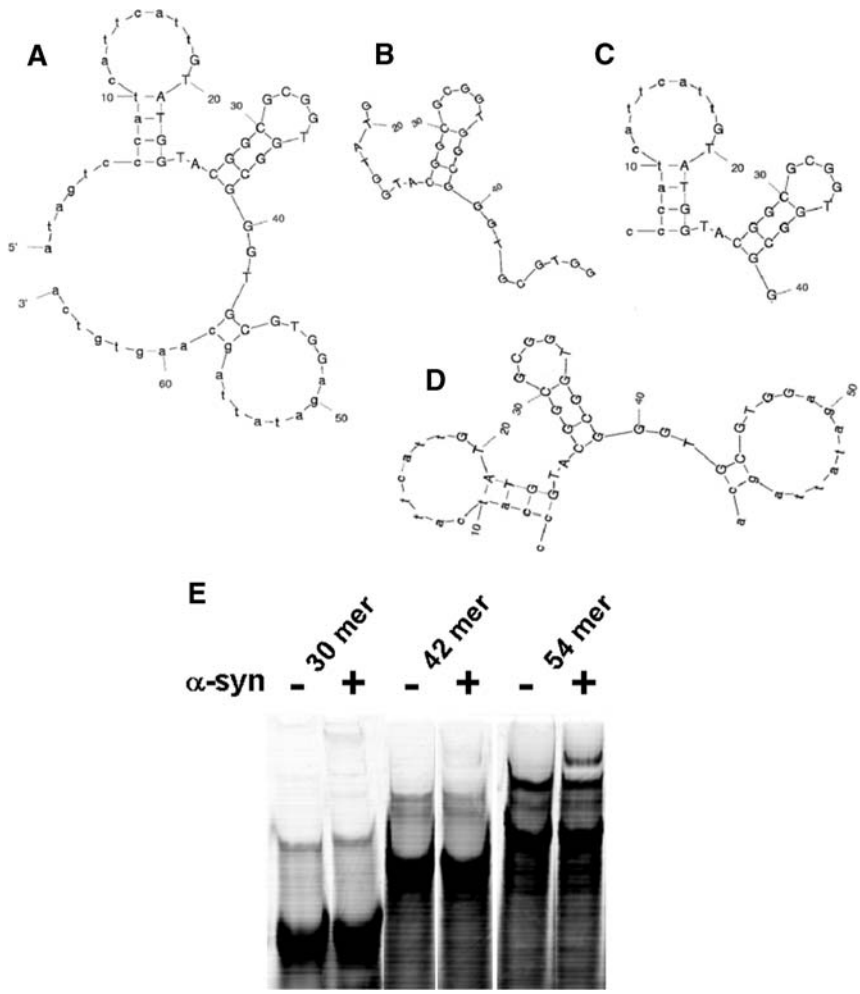

Fig. 2 Predicted secondary structure of M5-15 (**a**; 66 mer) and the truncated mutants (**b**; 30 mer, **c**; 42 mer, **d**; 54 mer). The structure of M5-15 was predicted by the M-fold program, and was determined to be the most stable according to the Gibbs free energy. **e** Binding of the truncated aptamers to α-synuclein. The plus and minus signs indicate that the aptamer was incubated with or without α-synuclein, respectively

(Fig. 2b). The second mutant had the 3′-end of the oligonucleotides removed from M5-15 and two stem-loop structures, and was 42 mer in length (Fig. 2c). The last one retained all the stem-loop but the extra regions were removed at both ends, and the length of this truncated mutant was 54 mer (Fig. 2d). These truncated mutants of M5-15 were analyzed in terms of their ability to bind to α-synuclein by gel shift assay (Fig. 2e). The mutants having one or two stem-loop structures had decreased binding capability. Therefore, M5-15 seemed to need at least three stem-loop structures to bind to α-synuclein.

Evaluation of the specificity of M5-15 for α-synuclein

To test whether M5-15 has specificity against α-synuclein, we used aptamer blotting with $A\beta_{1\text{-}42}$ and BSA as protein competitors. $A\beta_{1\text{-}42}$ forms the insoluble β-sheet conformation as well as α-synuclein, and the deposition of $A\beta_{1\text{-}42}$ correlates with Alzheimer's disease. Although an equimolecular amount of each protein was immobilized on the same membrane, only M5-15 bound to α-synuclein (Fig. 3). This result indicates that M5-15 can discriminate α-synuclein from $A\beta_{1\text{-}42}$ and BSA.

Evaluation of the binding of the aptamers to the α-synuclein monomer and oligomer using aptamer blotting

We evaluated the binding ability of M5-15 to the α-synuclein monomer and oligomer. Because α-synuclein oligomer consists of α-synuclein monomers, it is expected that M5-15 can bind to α-synuclein oligomer as well as to α-synuclein

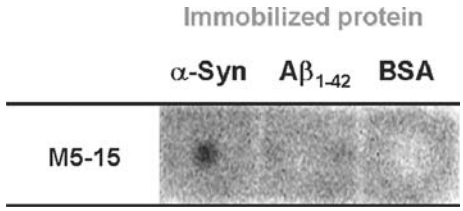

Fig. 3 Binding specificity of M5-15, as determined by the aptamer blotting assay. One hundred and forty picomole aliquots of α-synuclein, $A\beta_{1\text{-}42}$ and BSA were immobilized onto the same membrane

monomer. Immobilized α-synuclein monomer was detected by M5-15, and the detection chemiluminescence depended on the amount of immobilized α-synuclein (Fig. 4a). In addition, although the same amount of α-synuclein monomer and oligomer were immobilized within the same volume, the evaluation by aptamer blotting indicated that M5-15 bound more strongly to α-synuclein oligomer (Fig. 4b). Moreover, when we used 4 μg of monomer and 1 μg of oligomer, M5-15 appeared to bind to the α-synuclein oligomer, while the 66-mer thymine (PolyT) appeared not to bind to both α-synuclein forms (Fig. 4c). Therefore, we can state that M5-15 selectively binds to the α-synuclein oligomers.

Discussion

By using SELEX with native PAGE, we isolated a DNA aptamer that binds to α-synuclein. Aptamers are useful as molecular-recognition ligands, because they can be easily synthesized and modified, and they are amenable to designed changes in their structures. Several aptamers against proteins associated with neurodegenerative disorders have been developed for diagnostic and therapeutic purposes, so that α-synuclein-binding aptamers could be suitable for use in studies on neurodegenerative disease caused by α-synuclein.

We obtained several aptamers from the fifth library and found the M5-15 had the highest affinity for α-synuclein. The result of the gel shift assay indicates that this aptamer may have two states, one which binds to α-synuclein and one which does not. There is the similar report which one aptamer has two structures, and each structure have different functions (Huang et al. 2009). We then predicted the DNA structure with M-fold program and designed three truncated mutants which have different stem-loop structures. According to the binding ability of these truncated mutants as determined by the gel shift assay, M5-15 needs three stem-loop regions to recognize α-synuclein.

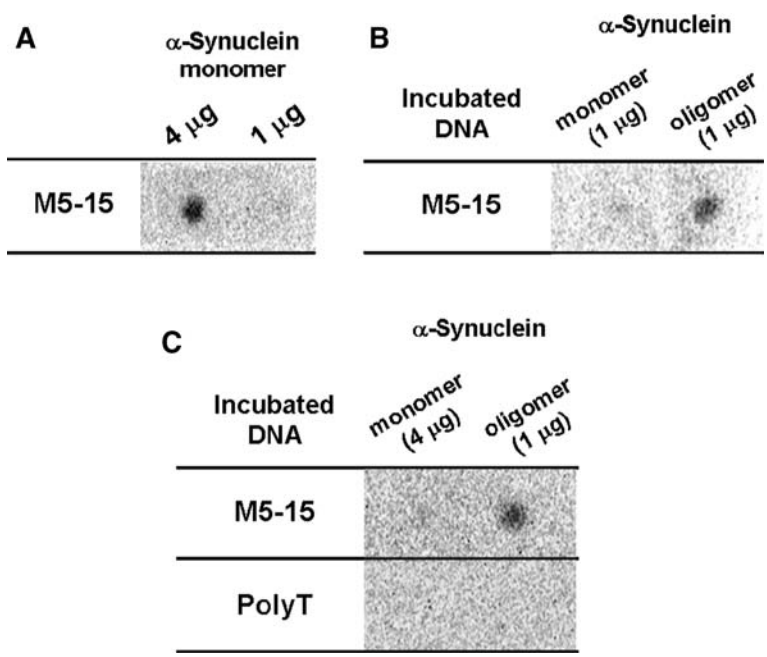

Fig. 4 Aptamer blotting assay to evaluate the ability of M5-15 to bind to the α-synuclein monomer and oligomer. **a** The membrane onto which 4 and 1 μg of α-synuclein were immobilized was incubated with 5 μM FITC-labeled M5-15. **b** The same volumes of α-synuclein monomer and oligomer were immobilized on the membrane. **c** A 4-fold quantity of α-synuclein monomer was immobilized on the membrane. PolyT (consisting of 66-mer thymine) was used as the control

We compared the binding capacities of M5-15 with α-synuclein, $A\beta_{1\text{-}42}$, and BSA. The central region of α-synuclein forms the non-amyloid β-component of Alzheimer's disease amyloid. BSA was used in this assay because of its low pI (4.7–4.9), which is similar to that of α-synuclein. The aptamer blotting assay showed that M5-15 bound only to α-synuclein indicating that M5-15 is specific for α-synuclein and can distinguish α-synuclein from other proteins associated with neurodegenerative diseases. This property of M5-15 will be very important in research on neurodegenerative diseases.

M5-15 bound not only to the α-synuclein monomer but also to the α-synuclein oligomer. Furthermore, M5-15 bound to the α-synuclein oligomer more robustly than to the α-synuclein monomer. There might be several reasons why M5-15 bound to oligomer compared with the monomer. Because α-synuclein has a native, unfolded structure, M5-15 may recognize certain regions of the α-synuclein amino acid sequence. Then, in the case of the evaluation of aptamer blotting, the aptamer recognizes sequences of α-synuclein that are not sufficiently exposed when α-synuclein monomers are immobilized. On the other hand, the α-synuclein oligomer is expected to form a rigid conformation in comparison to that of the α-synuclein monomer, which suggests that α-synuclein may exist in the oligomeric state when it is immobilized on the membrane. The results of aptamer blotting suggest that α-synuclein oligomers have aptamer-recognizing sequences on the surface, and that this conformation may remain unchanged even after immobilization on the membrane. This may be the reason why M5-15 binds selectively to the α-synuclein oligomer.

Recent research into neurodegenerative diseases has focused on the oligomer as the most important target because its toxicity is higher than that of fibrils. The screening of a novel DNA aptamer which binds to the α-synuclein monomer and oligomer may be helpful in the investigation of PD and for the development of new diagnostic and therapeutic tools.

References

Ding TT, Lee SJ, Rochet JC, Lansbury PT Jr (2002) Annular alpha-synuclein protofibrils are produced when spherical protofibrils are incubated in solution or bound to brain-derived membranes. Biochemistry 41:10209–10217

El-Agnaf OM, Salem SA, Paleologou KE et al (2006) Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. FASEB J 20:419–425

Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346:818–822

Huang Z, Pei W, Han Y, Jayaseelan S et al (2009) One RNA aptamer sequence, two structures: a collaborating pair that inhibits AMPA receptors. Nucleic Acids Res 37(12): 4022–4032

Kobayashi M, Kim J, Kobayashi N et al (2006) Pyrroloquinoline quinone (PQQ) prevents fibril formation of alpha-synuclein. Biochem Biophys Res Commun 349:1139–1144

Mathews DH, Sabina J, Zuker M et al (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 288:911–940

Ogasawara D, Hasegawa H, Kaneko K et al (2007) Screening of DNA aptamer against mouse prion protein by competitive selection. Prion 1:248–254

Paleologou KE, Kragh CL, Mann DM et al (2009) Detection of elevated levels of soluble alpha-synuclein oligomers in post-mortem brain extracts from patients with dementia with Lewy bodies. Brain 132:1093–1101

Proske D, Gilch S, Wopfner F et al (2002) Prion-protein-specific aptamer reduces PrPSc formation. Chembiochem 3:717–725

Rhie A, Kirby L, Sayer N et al (2003) Characterization of 2′-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion. J Biol Chem 278:39697–39705

Takahashi T, Tada K, Mihara H (2009) RNA aptamers selected against amyloid beta-peptide (Abeta) inhibit the aggregation of Abeta. Mol Biosyst 5:986–991

Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505–510

Weinreb PH, Zhen W, Poon AW et al (1996) NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. Biochemistry 35(43):13709–13715

Weiss S, Proske D, Neumann M et al (1997) RNA aptamers specifically interact with the prion protein PrP. J Virol 71:8790–8797

Ylera F, Lurz R, Erdmann VA, Furste JP (2002) Selection of RNA aptamers to the Alzheimer's disease amyloid peptide. Biochem Biophys Res Commun 290:1583–1588

Yoshida W, Sode K, Ikebukuro K (2006) Aptameric enzyme subunit for biosensing based on enzymatic activity measurement. Anal Chem 78:3296–3303

Yoshida W, Sode K, Ikebukuro K (2008) Label-free homogeneous detection of immunoglobulin E by an aptameric enzyme subunit. Biotechnol Lett 30(3):421–425