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An RNA aptamer that recognizes a specific conformation of the protein calsenilin

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Abstract—The generation of molecules that selectively recognize specific conformations of a protein is an important component of the elucidation protein function. We have used SELEX (Systematic Evolution of Ligands by EXponential enrichment) technology to produce aptamers that bind in a conformationally selective manner to calsenilin, which involved in Ca^{2+}-mediated apoptotic signaling. Since the conformations of calsenilin are quite different in the presence and absence of Ca^{2+}, aptamers were selected against the dimeric protein both under calcium-bound and calcium-free conditions. We have found that aptamer-12 selectively binds to the dimeric form of the protein in the presence of calcium ion, while the binding of aptamer-2 does not discriminate between the Ca^{2+} bound and unbound protein. Data obtained from biochemical and biophysical experiments suggest that a dominant conformation of calcium-bound calsenilin exists in one dominant conformation and that one aptamer can be generated to recognize this conformation. In addition, observation made in this effort that aptamers selected against the two different conformations of calsenilin have different characteristics suggest that aptamers can serve as a plausible tool for recognizing various conformations of proteins, even those caused by interactions with small molecules or ions such as Ca^{2+}.

1. Introduction

Calsenilin is a protein that interacts with presenilin-2 to cause production of pathogenic Aβ42 in Alzheimer's disease (AD).[1] The protein is active when calcium ion is bound to its EF-hand domain.[2] Increased intracellular calcium concentrations favor the active conformation, which induces neuronal apoptosis through interaction with the C-terminus of presenilin-2.[3] Calsenilin is known by other names, for example, Kv channel-interacting protein 3 (KChIP3) and downstream regulatory element antagonist modulator (DREAM), owing to its functions in different cellular locations.[4] These functions are also governed by calcium ion, suggesting that active conformation(s) of the protein is modulated by binding of Ca^{2+}.[5]

As a functional protein, the biochemical role played by calsenilin may be a consequence of its different conformations. Intrinsically flexible proteins have several energetically degenerate conformations and the active conformation must be induced by binding of a signaling molecule. In the case of calsenilin, calcium ion is the signaling agent.[6] The signaling activity of this protein is then mediated by formation of this active conformation.[7] In order to obtain a detailed picture of the biochemical role of this protein, it is important to gain information about its active conformation(s).

One approach to this problem is to develop conformationally specific antibodies. These can be generated and used among naïve repertoires of genes, but only with low probability.[8] The generation of highly probable repertoires of antibody genes against a certain specific conformation of a protein is a difficult task because a conformational dominance of the protein cannot be maintained during the long period needed for

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immunization. On the other hand, conformationally specific aptamers can be selected by using a biased condition in which one protein conformation can be made to dominate over others by using small molecules or ions.[9] In spite of its logical simplicity, only few examples of conformationally specific aptamers have been developed by using this strategy[10] even though it possesses an economical advantage over an antibody based approach.

Below we describe the results of a recent study which demonstrates that (1) the selection of aptamers against calsenilin both in calcium-bound and calcium-free conformations can be made by using a standard SELEX procedure, and (2) the selected aptamer discriminates between Ca^{2+}-bound and -unbound conformations of the protein. The findings suggest that aptamers can serve as conformationally selective capturing molecules for various functional proteins.

2. Results and discussion

2.1. Selection of RNA against the biased conformation(s) of calsenilin

Initial experiments were designed to determine if conformational preferences for calsenilin can be induced by interactions with a fusion protein and calcium ion. For this purpose, calsenilin was expressed in a GST-fused form (see Section 4) to promote formation of dimeric conformations. Circular dichroism (CD) spectra of the protein in the presence and absence of Ca^{2+} were recorded. A spectra, displayed in Figure 1a, show that calcium binding causes an increase in the α-helical content of calsenilin as is observed with other EF-hands-containing proteins.[11] The observation of fluorescence intensity changes caused by bis-ANS binding to the protein adds further support for the proposal that conformational changes take place when calsenilin binds calcium ions. In this case, the intensity of fluorescence decreases with increasing concentration of Ca^{2+} concentration (Fig. 1b). The fluorescence assay data suggest that calcium ion induced conformational changes in the protein lead to a reduction in the hydrophobic surface area.[12] In addition, size exclusion chromatographic (SEC) analysis (Fig. 1c) shows that the retention time of the fusion protein is increased in the presence of calcium ion as compared to that under calcium-free conditions. This result indicates that a more compact conformation is formed when calsenilin binds calcium ion.[13]

Calsenilin is known to exist in a dimeric active conformation in neuronal cell death in the presence of Ca^{2+}.[1,3–5] Consequently, a standard SELEX technology was carried out[14] using GST-fused calsenilin throughout the whole selection cycle.[15] Since dimeric calsenilin

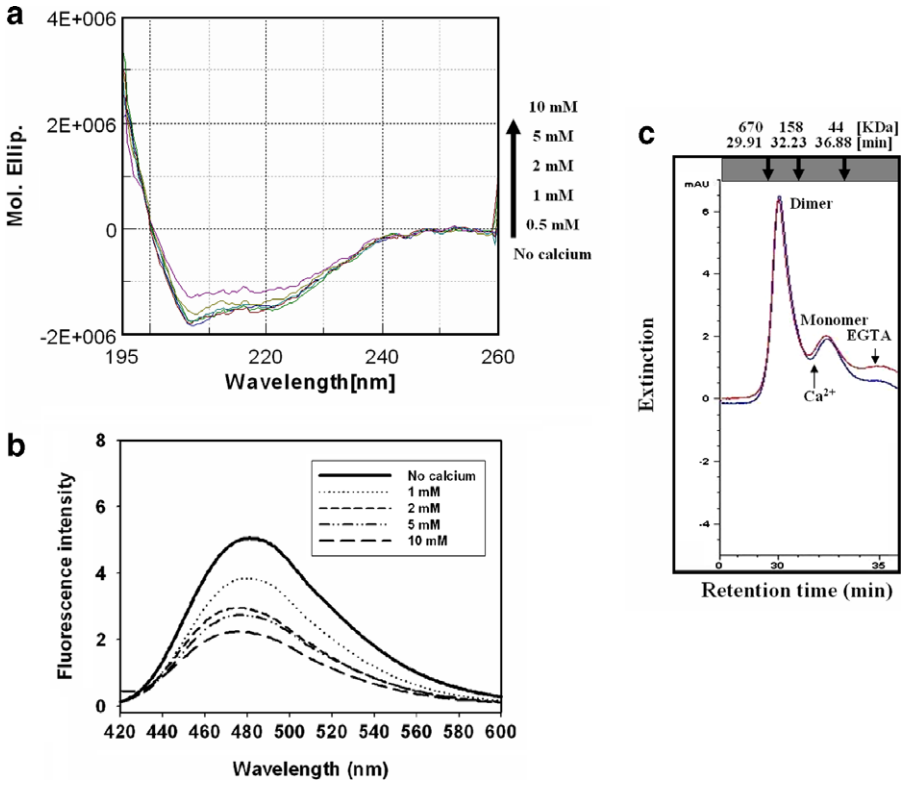

Figure 1. Conformation studies of calsenilin protein. (a) CD spectra of recombinant calsenilin protein. GST-calsenilin protein (3.65 μM) was used. Various concentrations of calcium (0.5–10 mM) were applied. (b) Bis-ANS fluorescence spectra of recombinant calsenilin protein. GST-calsenilin (7.3 μM) was mixed with bis-ANS (7.3 μM) and then various concentrations of calcium (1–10 mM) ion were applied and fluorescence spectra were recorded. (c) SEC of GST-fused calsenilin protein. Measurements were performed on a Shodex protein KW802.5 column at room temperature. Elution profiles of GST-calsenilin protein (1 μM) in the presence or absence of calcium ion were shown. GST-calsenilin protein is predominantly dimer. Furthermore, elution time of calcium-free conformation of GST-fused calsenilin was shifted to high molecular weight elution time. Molecular masses (KDa) and elution times (min) of standard protein are shown on top.

Table 1. Sequences of selected RNA clones[a]

	Sequences of the random region
Group I	#2: GACGGAGGCT TGTTTATGTA GGGATGTAAG GGGATGGGCA ATGTGGCGAC
	#9: GACGGAGGCA TGTCTATGTA GGGACGTAAG GGGATGGGCA ATGTGGCGAC
	#13: GACGGAGGCA TGTTTATGTA GGGACGTAAG GGGATGGGCA ATGTGGCGAC
	#23: GACGGAGGCTTTGTTTATGTA GGGACGTAAG GGGATGGGCA ATGTGGCGAC
Group II	#3: TTAGGAATGG CGGGTCGAGG AATAAGAGCC CATGATAGCC TTTGGTGCA
	#7: TTAGGAATGG CGGGTCGTGG AATAAGAGCC CATGATAGCC TTTGGTGCA
	#24: TTAGGAATGG CGGGTCGAGG AATAAGAGCC CATGATAGCC TTTGGTACT
Group III	#8: AAAGAGGCTG CGCGGAAGTG AGGGTGTTGG TTACGAAGGT TGGTGTGTA
	#12: AAAGAGGCTG CGCGGAAGTG AGGGTGTTGG TTACGAAGGT TGGTGTGTGA

[a] 5′-Fixed region: GGGACGCGTGGTACC, 3′-fixed region: AGCTT CCGCGGGGATC.

adopts different conformations in the presence and absence of calcium,[16] aptamer selections were independently carried out under both calcium-rich and calcium-free conditions. Selected RNA pools, obtained following 9 cycles of the SELEX procedure in the presence or absence of calcium ion, were cloned and sequenced. Sequences of the selected aptamers were divided into three groups (Table 1) in which members of groups I and II derive from calcium-free conditions and of group III come from calcium-bound conditions. Each aptamer clone was subjected to the pull-down assay to confirm its binding selectivity (Fig. 2). Each cloned aptamer do not bind to fused GST domain. Aptamer-2 from group I was found to strongly bind to the GST-fused calsenilin both in the absence and presence of calcium ion. Aptamer-3 from group II had no preference for calsenilin conformations either, but it had a weaker binding affinity than aptamer-2. Interestingly, aptamer-12 from group III had a strong binding affinity to the protein only in the presence of calcium ions. These

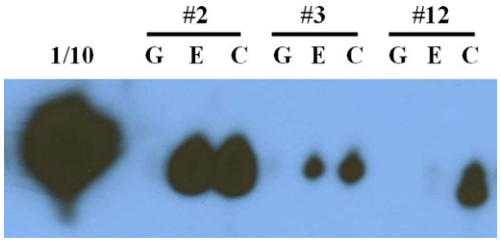

Figure 2. Binding selectivity test of selected aptamer clones using pull-down assay. After 9 cycles of selection, selected clones were tested by pull-down assay in the presence or absence of calcium. Five hundred picometers of 5′-^{32}P end-labeled RNA was used. Aptamer-2 was bound to each conformation of calsenilin with strong binding affinity. Interestingly, aptamer-12 was bound to only calcium-bound conformation of calsenilin protein (1/10, 1/10 input RNA; G, GST 500 nM; E, GST-calsenilin 500 nM in EGTA buffer; C, GST-calsenilin 500 nM in calcium buffer).

observations suggest that this substance could be a 'conformationally specific aptamer'.

2.2. Evaluation of selected RNA as conformation-specific aptamers

Owing to the fact that aptamer-2 displays the strongest binding and aptamer-12 is the most specific binding to GST-fused calsenilin, both were subjected to more detailed study. Surface plasmon resonance (SPR) assays were carried out with these aptamers in order to determine their binding affinities and specificities more accurately. The injections of various concentrations of each aptamer through the immobilized protein were carried out to calculate binding affinities. As the data in Figure 3 demonstrate, aptamer-2 has nearly the same exceeding low dissociation constants with both the Ca^{2+}-bound ($K_D = 4.3 \times 10^{-8}$ M) and Ca^{2+}-unbound protein ($K_D = 7.9 \times 10^{-8}$ M). Aptamer-12, which was shown by the pull-down assay procedure to bind selectively to only the Ca^{2+}-bound state of calsenilin, more weakly binds to the protein ($K_D = 1.9 \times 10^{-7}$ M) than does aptamer-2. Importantly, this aptamer does not bind at all to the protein under Ca^{2+}-free conditions. In addition, we observed that, unlike aptamer-12, the commercially available anti-calsenilin polyclonal antibody (Invitrogen) does not display selective binding to calsenilin (Data not shown). Rather, the results of the SPR assay show that this antibody strongly binds to both the calcium-bound ($K_D = 1.3 \times 10^{-8}$ M) and calcium-free dimeric protein ($K_D = 5.4 \times 10^{-9}$ M).

An electrophoretic gel mobility shift assay was carried out to confirm the specificity of aptamer binding to dimeric calsenilin (Fig. 4). As expected, a band associated calcium bound calsenilin complexed with aptamer-12 was only observed by using this procedure. Bands were observed for aptamer-2 complexed with calsenilin in both the presence and absence of Ca^{2+}. The data suggest that aptamer-2 recognizes a general epitope of the protein and that it cannot discriminate between various conformations of the protein. A possible reason for the lack of specificity of aptamer-2 is that the protein, under calcium deficient condition, does not exist in a single dominant conformation. This reasoning gains support from the results of previous studies, in which a broad elution pattern was observed in the SEC of apo-calsenilin indicating a folded yet flexible molten globule-like structure of metal-free calsenilin.[2,5] When bound to calcium, on the other hand, the protein forms a dominant and active conformation which is reflected in aptamer-12 binding.

2.3. Mapping calsenilin binding regions on selelcted RNA aptamers

RNA footprinting experiments were conducted to map epitopes of aptamers selected against the protein. RNAse protection regions of the two aptamers were found to be located mainly on loops, shown as lined regions in Figure 5a and b for aptamer-2 with calcium-rich and -deficient calsenilin, respectively, or in Figure 5c for aptamer-12 with calcium-rich calsenilin. Loops are

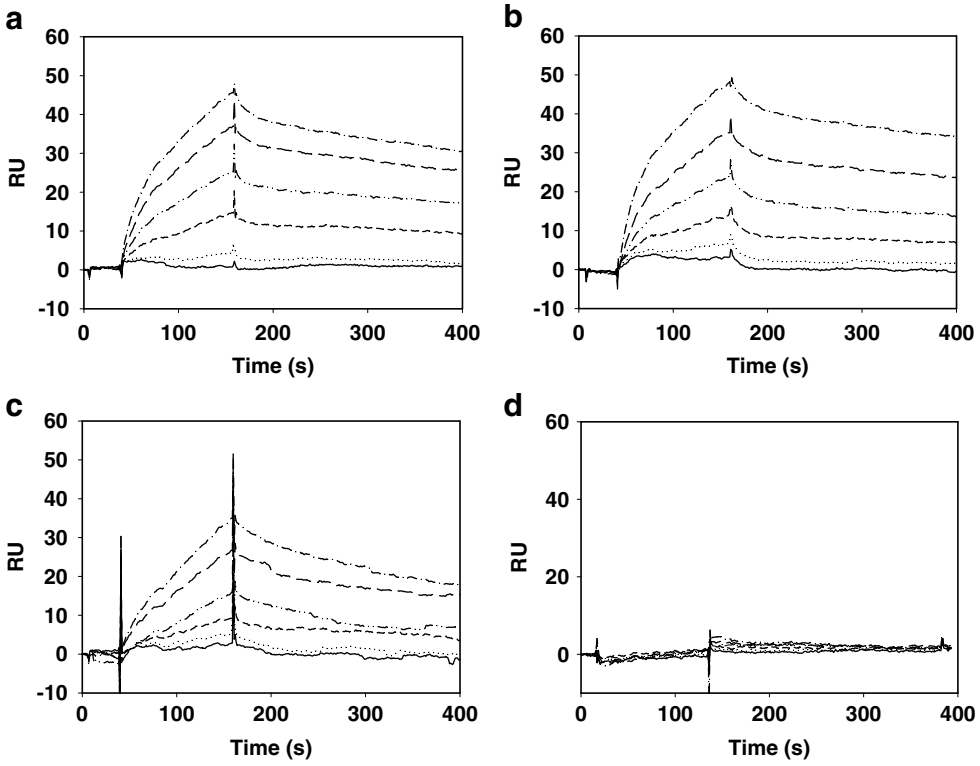

Figure 3. Binding specificity of aptamer-2 and -12 using surface plasmon resonance (SPR) techniques. (a) and (b) Sensorgrams of aptamer-2 binding to calcium-bound and calcium-deficient conformations, respectively. (c) and (d) Aptamer-12 binding to calcium-bound and calcium-deficient conformations, respectively. Various concentrations of aptamers were injected (31.25, 62.5, 125, 250, 500 nM, and 1 μM). RU, response unit.

typical motifs that are responsible for recognition of protein targets.[17] The protection regions of aptamer-2 (31A to 60G in Fig. 5b) in the absence of Ca^{2+} ion are extended to a neighboring bulged loop (AUGU sequences in the triangle region in Fig. 5g) in contrast to those protected in the presence of the calcium ions (35A to 60G in Fig. 5a). The protection region in aptamer-12 was from 37G to 68G in the presence of calcium ions (Fig. 5c). In order to obtain information about more accurate binding regions, we designed the truncated RNA aptamer-2 and -12 containing the minimal binding domains. These regions were presented as lines in Figure 5a–c, structures of them were depicted in Figure 5g and h. As shown in Figure 5d–f, RNase protection of truncated aptamers once again confirmed that these stem-loop structures are minimal binding domains required for calsenilin binding.

The data taken from RNA footprinting using full-length and truncated aptamers suggest that aptamer-2 is capable of recognizing at least two different conformations of calsenilin in the presence and absence of calcium ion. A more extended epitope might be suitable to recognize more relaxed conformations of the protein under calcium-free conditions, while a less extended epitope might be able to recognize more compact conformation

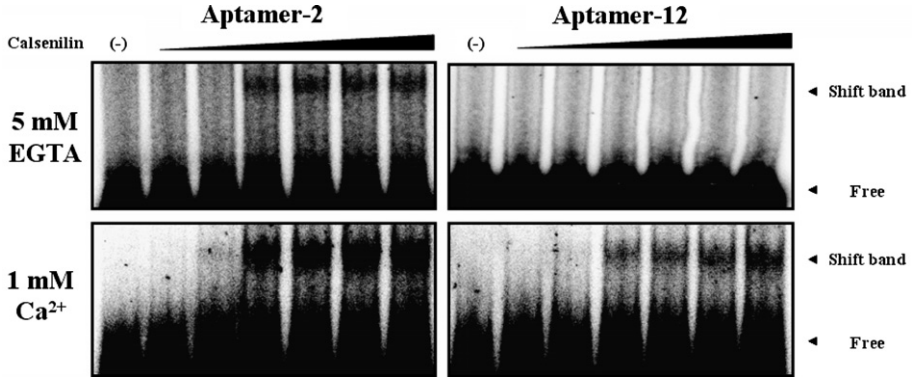

Figure 4. Determination of binding preferences of aptamers for dimeric calsenilin conformations using electrophoretic mobility shift assay. Fifty nanometers of 5′-^{32}P labeled each aptamer and various concentrations of calsenilin protein (0, 100, 200, 400, 600, 800 nM, and 1 μM) were incubated in each sample. Samples were electrophoresed on 4% native gel in calcium-deficient or calcium-rich condition.

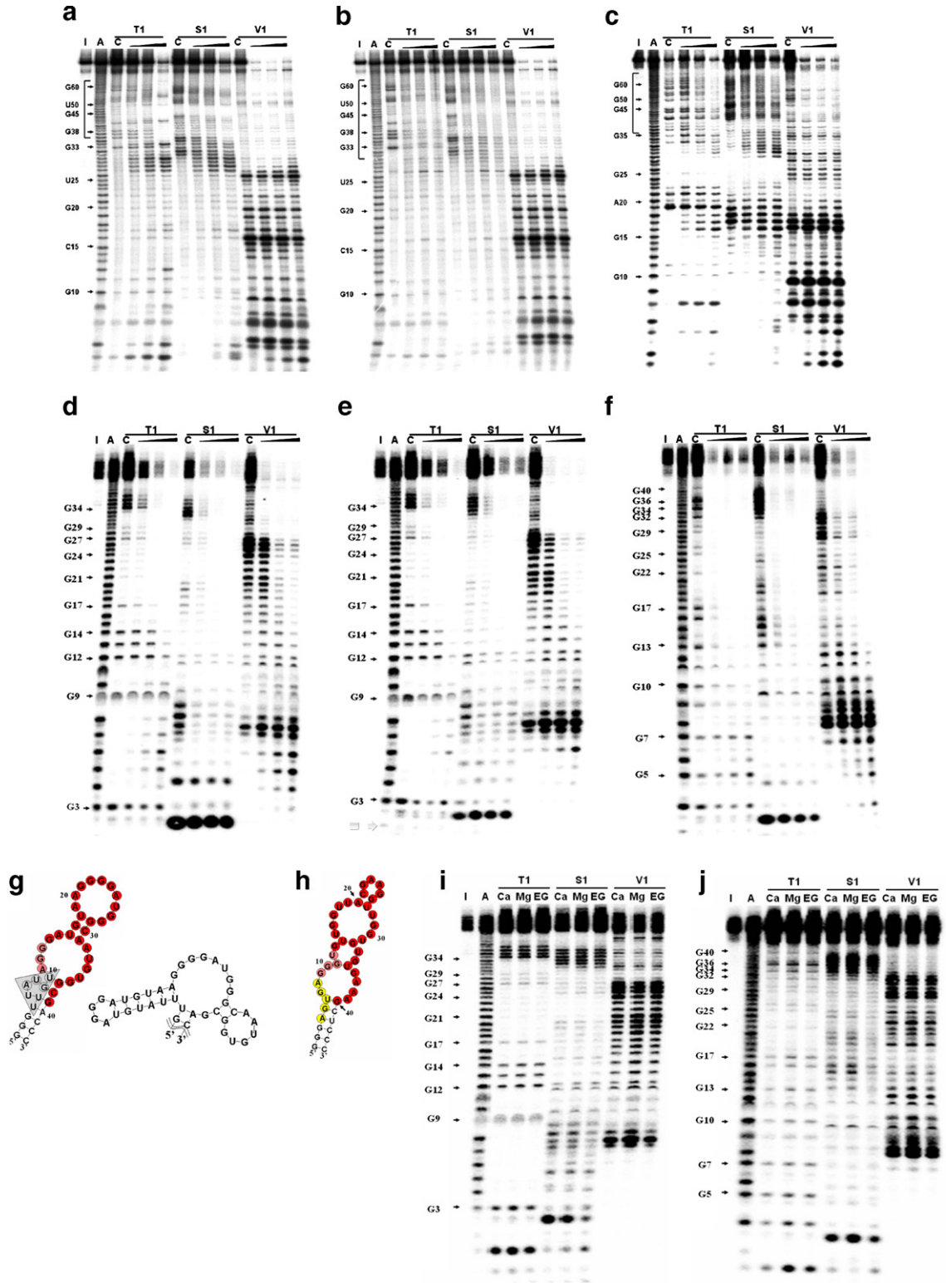

Figure 5. Determination of calsenilin-binding regions of aptamer-2 and -12 using RNA footprinting assay. (a) and (b) Autoradiograms of aptamer-2 in calcium-rich and calcium-deficient conditions, respectively, against calsenilin. (c) Autoradiogram of aptamer-12 in calcium-rich condition against calsenilin. Lined sequences indicate the minimal binding domains of RNA aptamers. Two hundreds nM of 5′-^{32}P labeled RNA was used for each lane. Various concentrations (100-, 250-, 500-fold to RNA) of calsenilin were applied. (d), (e), and (f) The same systematic experiment was carried out in sequence as described in (a), (b), and (c), except for using of truncated RNA aptamers. (g) Calsenilin-binding region on aptamer-2. The most stable structure was shown on the left side and the other less stable structure was shown on the right side. RNA secondary structure was predicted using Michael Zuker's m-fold program on http://www.bioinfo.rpi.edu/~zukerm. (h) Calsenilin binding region on aptamer-12. (i) and (j) Metal ion dependency of RNA aptamer-2 and -12 structure, respectively (A red region, strong protection region; A pink region, weak protection region; A yellow region, enhanced cleavage region; A triangle region, extended binding region for calcium-deficient calsenilin; I, intact aptamer control; A, alkaline hydrolysis control; C, no recombinant calsenilin; T1, RNAse T1; **S1**, RNAse S1; V1, RNAse V1; Ca, calcium buffer; Mg, magnesium buffer; EG, EGTA buffer).

of the calcium bound protein. An M-FOLD prediction applied to aptamer-2 yielded several energetically degenerate secondary structures, suggesting several conformations could coexist. One of them containing different secondary structure on a minimal binding domain was shown in Figure 5g. This finding supports the proposal that aptamer-2 possesses a pliable structure that can adapt to the conformation of the protein that it encounters. In contrast, only one M-FOLD predicted secondary structure is found for aptamer-12, and this structure uniquely recognizes the stable conformation of the protein formed in the presence of Ca^{2+}.

Like conformations of proteins are induced by calcium ion, those of aptamers could be influenced by Ca^{2+}, Mg^{2+}, and other divalent ion. In order to defy the possibility, we carried out RNA footprinting experiments with the selected aptamers in the presence and absence of divalent ion. Two autoradiograms of RNA aptamer-2 and -12 (Fig. 5i and j, respectively) with two different divalent metal ions (Ca and Mg lanes, respectively, in Fig. 5i and j) and without them (EG lanes) gave identical cleavage patterns by various RNases. This result suggests that the most stable conformations of both RNA aptamers are independent on divalent ions. Therefore, there is no conformational change of RNA aptamer-12 by calcium ions. Aptamer-12, per se, is a conformational specific antibody-like ligand that can discriminate calcium-induced and calcium-independent conformations of the target protein.

3. Conclusion

The results of this investigation demonstrate that antibody-like aptamers can be selected to selectively bind to different conformations of calsenilin. Specifically, by using GST-fused calsenilin in the presence and absence of Ca^{2+}, we were able to generate the corresponding active and inactive conformation of the protein. Aptamer-12, selected for the active conformation of the protein, binds strongly to the calcium-bound dimeric conformation and not to the calcium-deficient conformation (i.e., it has a specific epitope that selects for the active form of the calsenilin protein). This is the case even though aptamer-12 displays *ca.* 15- to 35-fold weaker binding affinity to the protein in comparison to that of antibodies. The overall significance of the effort comes from the finding that aptamers can be superior to antibodies in recognizing specific, active conformations among the many that are possible for proteins in living systems.

4. Experimental

4.1. Purification of calsenilin

Full length calsenilin (Mus musculus calsenilin/DREAM,[18] AAF14576 as accession number in NCBI protein database, 256 amino acids) fused to glutathione S-transferase construct (pGEX-4T-calsenilin) was kindly provided by Prof. Yong-Keun Jung (Seoul National University). The GST fusion form of calsenilin was overexpressed in *Escherichia coli* (*E. coli*) BL21 and was purified by glutathione–sepharose 4B (Amersham).

4.2. Circular dichroism

Circular dichroism (CD)[19] spectra were recorded on a Jasco J-715 spectrapolarimeter at room temperature. Far-UV CD of GST-calsenilin protein (0.2 mg/mL) was performed in 5 mM Tris, 50 mM NaCl pH 7.5. To study the conformational change of calsenilin by calcium ion, various concentrations (0.5–10 mM) of calcium were applied.

4.3. 4,4′-Dianilino-1,1′-dinaphthyl-5,5′-disulfonic acid (bis-ANS) fluorescence binding assay

Bis-ANS fluorescence binding assay was carried out with Aminco Bowman series 2 luminescence spectrometer. Excitation and emission wavelength of bis-ANS was 395 nm and 500 nm, respectively. Band-pass was 8 nm and fluorescence scanning was carried out at 25 °C. GST-calseniln (7.3 μM, 500 μL) protein was mixed with bis-ANS fluorescene dye (7.3 μM, 500 μL) in 25 mM Tris, 100 mM NaCl, pH 7.5. To study the conformational change of calsenilin by calcium ion, various concentrations (0.5–10 mM) of calcium were applied. GST signal was normalized by subtracting with data of GST sample.

4.4. Size exclusion chromatography

Size exclusion chromatography (SEC) was carried out on a Shodex protein KW802.5 column at room temperature in the binding buffers containing 1 mM of $CaCl_2$ or 5 mM of EGTA as described above. Agilent 1100 HPLC system was used for this assay and a flow rate was 0.2 mL/min. Gel filtration standard mixture (100 μL, Bio-Rad) was loaded as a molecular mass standard of the protein. GST-fused (1 μM) was incubated in each binding buffer for 15 min at room temperature. Then, samples were loaded on a column at 25 °C and spectra were recorded at 280 nm wavelength.

4.5. In vitro selection

A DNA library containing random sequences of 50 nucleotides[20] was synthesized by Integrated DNA Technology (USA). The random region was flanked by defined sequences containing the T7 promoter and restriction enzyme sites for in vitro transcription and cloning [5′-CGGAATTCGTAATACGACTCACTA TAGG GACGCGTGGTACC(N50)AAGCTTCCGCG GGGATCCAA-3′]. The DNA library was amplified by general PCR condition [95 °C 5 min,(95 °C 30 s, 55 °C 30 s, 72 °C 30 s) 10 cycles, 72 °C 8 min 30 s] using 5′-primer (5′-CGGAATTCGTAATACGACTCACTATAG GGACGCGTGGTACC-3′) and 3′-primer (5′-TTGGA TCCCCGCGGAAGCTT-3′). The RNA library was prepared by in vitro transcription using T7 polymerase [40 mM Tris (pH 7.9 at 25 °C), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine]. Prepared RNA library con-

taining 1×10^{14} different molecules was used for first cycle. In each cycle, in order to remove GST or bead binders, pre-clearing step was carried out. Then, RNA molecules were incubated with 10 μg of GST-calsenilin in 200 μL of binding buffer [30 mM Tris (pH 7.5 at 25 °C), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, or 1 mM $CaCl_2$] with 40 U of RNase inhibitor for 30 min at room temperature. Glutathione–sepharose 4B (30 μL) was added to the sample and then the sample was subjected to shaking for 30 min at room temperature. Protein-RNA complexes were pelleted and washed three times with binding buffer (500 μL). And then, RNA was eluted by incubating with 5 mM EDTA for 5 min at room temperature. The resulting mixture was subjected to phenol/chloroform extraction for eluting RNA aptamers from calsenilin proteins bound to beads. The eluted RNAs were purified by micro-spin G-25 column (Amersham) and ethanol precipitation. It was reverse transcribed by M-MuLV reverse transcriptase (Roche) using 3′ primer (500 μM) and each of dNTPs (1 mM) and amplified by 20 cycles of PCR. The amplified DNA was then transcribed into RNA followed by next selection cycle as described above. After 9 cycles of selection, the selected and amplified DNA was inserted into pUC19 vector using EcoRI, BamHI restriction site, and T4 DNA ligase. The recombinant DNA was transformed into *E. coli*. Plasmid DNA was prepared from individual clones and sequenced.

4.6. Pull-down assay

Pull-down assay was carried out with same procedure as described above (selection step in SELEX cycle). Briefly, 500 pM of 5′-^{32}P end-labeled RNA was mixed with 500 nM of GST or GST-calsenilin protein in binding buffer for 30 min at room temperature. Glutathione sepharose 4B was added to each solution and incubated for 30 min at room temperature with gentle shaking. The resulting suspensions were centrifuged and the supernatant containing unbound RNA was discarded. The pelleted beads were washed three times with binding buffer. Bound RNA was eluted with 5 mM EDTA for 5 min at room temperature, and fractionated and analyzed by 6% polyacrylamide/7 M urea gel electrophoresis and autoradiography.

4.7. Surface plasmon resonance (SPR) assay[21]

Assay was carried out on a BIAcore 3000 instrument at room temperature. GST-calsenilin protein was added in binding buffer containing 10 mM $CaCl_2$ or 5 mM EGTA to make up 112 μg/200 μL stock solution. After 30 min incubation at room temperature, the stock solution was diluted to 1/10 in 10 mM sodium acetate (pH 5.0). The flow rate was adjusted as 5 μL/min. The carboxymethyl dextran matrix of CM5 sensor chip was activated by injection of 35 μL of coupling solution [200 mM *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide, 50 mM *N*-hydroxysuccinimide]. The diluted calsenilin solution was then injected into the activated flow cell. Unreacted NHS esters were deactivated by injection of ethanolamine (35 μL of 1 M at pH 8.5), affording a surface that gave a final change in response units (RU) of GST (as reference) and GST-calsenilin were 2100 and 4100, respectively. For the binding assay, flow rate was adjusted as 30 μL/min. Each aptamer or calsenilin antibody (Invitrogen) at various concentrations (31.25, 62.5, 125, 250, 500 nM, and 1 μM for aptamer, 3.94, 7.88, 15.75, 31.25, 62.5, and 125 nM for antibody) was injected by using the serial automated method which is comprised of sample injection (60 μL), dissociation (240 s), and regeneration (10 μL of 20 mM NaCl, 0.2 mM NaOH). The signals from a reference flow cell (GST-coupled chip surface) were subtracted from raw data. Binding affinity of each aptamer to calsenilin protein was calculated by using BIAevaluation software.

4.8. 5′-End labeling of RNA

The RNA was radio-labeled for the assay. Briefly, a mixture of 10 μg of RNA and 10 U of phosphatase (New England Biolabs) in 200 μL of reaction buffer [50 mM Tris (pH 7.9 at 25 °C), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT] was incubated for 1 h at 37 °C. RNA was then purified by phenol/chloroform extraction and ethanol precipitation. Next, a mixture of ^{32}P-labeled γ-ATP (Amersham Pharmacia Biotech) and the purified RNA was incubated with 50 U of T4 polynucleotide kinase (New England Biolabs) in reaction buffer [70 mM Tris (pH 7.6 at 25 °C), 10 mM $MgCl_2$, 5 mM DTT] for 1 h at 37 °C. The resulting RNA was electrophoresed on 6% acrylamide-7 M urea gel. The targeted RNA size was cut from the gel and eluted with elution buffer [0.5 M ammonium acetate, 1 mM EDTA, 0.1% (w/v) sodium dodesyl sulfate (SDS)], followed by ethanol precipitation and then the RNA was recovered by centrifugation.

4.9. Electrophoretic gel mobility shift assay

Fifty nanometers of 5′-^{32}P end-labeled RNA aptamers in binding buffer containing 1 mM $CaCl_2$ or 5 mM EGTA was heated at 65 °C for 5 min and cooled down to room temperature. Various amounts of calsenilin protein (100 nM–1 μM) containing competitor tRNA mixture (500 nM) were added to the prepared RNA solution and incubated for 30 min at 4 °C. Then RNA loading dye (2.5 μL, 50% glycerol in 22.5 mM Tris–borate, 0.5 mM EDTA buffer) was added to each sample. Resulting samples were electrophoresed on 4% native gel containing 4% (v/v) glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, or 5 mM EGTA with cooling. Running buffer was TBE (45 mM Tris–borate, 1 mM EDTA) buffer in the presence or absence of $CaCl_2$ (1 mM) for each condition.[22] The gel was dried and radioactivity of the individual bands was monitored by a phosphorimager (FLA-3000) and was analyzed by Multi Gauge Ver. 3.0 software (Fuji Photo).

4.10. RNA footprinting assay

Two hundred nanometers of 5′-^{32}P end-labeled RNA aptamers in binding buffer containing $CaCl_2$ (1 mM) or EGTA (5 mM) were heated at 65 °C for 5 min and cooled down to room temperature. Various amounts

of GST-calsenilin protein (100-, 250-, 500-fold concentration of RNA) were added to the prepared RNA solution and incubated for 20 min at room temperature. To the resulting solution, 1 μL of RNase (0.01 unit/μ L of RNase T1, 1 U/μL of RNase S1, or 0.001 U/μ L of RNase V1) was added and incubated for 10 min at 37 °C. Then fragmented RNA was obtained by ethanol precipitation. Resulting RNA was loaded on 15% polyacrylamide/7 M urea gel. The gel was dried and radioactivity of the individual bands was monitored by a phosphorimager (FLA-3000) and was analyzed by Multi Gauge Ver. 3.0 software (Fuji Photo).

Acknowledgments

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