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Binding of 14-3-3 proteins to a single stranded oligodeoxynucleotide aptamer

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Abstract

A synthetic library of *ca.* 10^{13} single stranded oligodeoxynucleotides, each comprising a randomized 40mer sequence and homogeneous 10mer flanking regions, was screened for binding to recombinant human 14-3-3γ. A single aptamer, which showed similar affinities ($K_D \sim 10^{-8}$ M) for six isoforms of the protein, has been shown to bind to undenatured 14-3-3 protein in the cerebral spinal fluid of scrapie infected sheep.

Keywords: Aptamer; 14-3-3; TSE; CJD; Affinity; Protein–DNA interaction

1. Introduction

The 14-3-3 proteins, a family of seven structurally similar protein isoforms which occur as both homo- and hetero-dimers, are known to bind to a large number of diverse protein targets and to play a multiplicity of roles in the control of cellular metabolism. These include the modification of the ligand binding efficiency of target proteins, the control of interorganelle protein trafficking and the regulation of catalytic activity [1–4].

While in healthy mammals 14-3-3 proteins are absent from the cerebral spinal fluid (CSF), varying amounts of 14-3-3 isoforms accumulate in the CSF of patients with neurodegenerative diseases [5–7]. 14-3-3 proteins are also detected in the CSF of sheep with scrapie and in cattle with bovine spongiform encephalopathy (BSE) [8]. Detection of 14-3-3 proteins is used in the diagnosis of sporadic Creutzfeld–Jacob disease (sCJD) in humans [9–11].

Development of a robust ELISA for 14-3-3 in CSF is complicated by its formation of complexes with its metabolic partners and the fact that, in its homo- and heterodimeric forms, [12–14] the distinctive N-terminal epitopes of the isoforms [5] are not completely exposed. As a result, the currently accepted protocol for detection and measurement for diagnostic purposes involves denaturation and Western blotting. It is well established that short nucleic acid sequences (aptamers), which exhibit protein-ligand affinities comparable to those with antibodies, can be selected from suitably large synthetic libraries [15–17]. Here we describe identification of a synthetic ssDNA aptamer which binds to undenatured 14-3-3 proteins and its application to the affinity precipitation of 14-3-3 from the CSF of a scrapie infected sheep.

2. Experimental

2.1. Cloning, protein expression and purification

The human 14-3-3γ gene was amplified from the GST fusion plasmid pGEX-4T1/14-3-3γ by PCR using the

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forward primer, 5′-GATCGGATCCGTGGACCCCGAG CAACTGGT-3′ (incorporating a BamHI restriction site), and the reverse primer 5′-GATCGTCGACTTAGTTG TTGTTGCCTTCTC-3′ (with a SalI site). The PCR product was ligated into the N-terminus His_6 tag expression vector pQE-30. Optimum protein expression was obtained using the BL21 (DE3) strain (Novagen) grown in 2YT broth containing ampicillin (100 μg/ml) and induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 3 h.

Two liters of culture grown under these conditions were harvested by centrifugation at 5000*g* for 15 min at 4 °C. The cell pellet was resuspended in 30 ml of resuspension buffer [Tris–Cl (50 mM, pH 7.5), Triton X-100 (0.05% w/v), imidazole (20 mM), and one Complete™ Proteinase Inhibitor Cocktail tablet (Roche) per 200 ml] at 4 °C. The cells were disrupted by sonication (10 pulses of 20 s with 20 s intervals) at 4 °C and cell debris removed by centrifugation (15,000*g*, 20 min, 4 °C). The supernatant was passed through a 0.45 μM membrane into a suspension of nickel nitrilotriacetic acid (Ni–NTA) superflow beads (Qiagen) in binding buffer [Tris–Cl (50 mM, pH 7.5), Triton X-100 (0.05% w/v), NaCl (0.5 M), imidazole (20 mM)]. The beads were loaded onto an empty polypropylene column (Bio-rad), allowed to drain and washed with 10 column volumes (50 ml) of the binding buffer. Bound proteins were eluted with elution buffer [20 ml, Tris–Cl (50 mM, pH 7.5), Triton X-100 (0.05% w/v), Imidazole (250 mM)], the eluate was diluted with an equal volume of sterile phosphate buffered saline (PBS, 50 mM phosphate, 20 mM NaCl, pH 7.4), dialysed into PBS using snakeskin membrane (Pierce) (MW 15 kDa) and stored at 4 °C. The His_6 14-3-3γ protein obtained (28 mg) proved essentially homogeneous by SDS and native gel electrophoresis. Electrospray LC-MS (Micromass Platform spectrometer coupled to a Waters 2690 HPLC using a Jupiter 5 μg C-5 300A column eluted with a linear gradient of 10–100% acetonitrile in water containing 0.01% TFA over 40 min at a flow rate of 0.05 ml/min) revealed the presence of one species with a molecular weight 29639.8 ± 2.3, corresponding to full length 14-3-3γ with the insertion of the sequence RGSHHHHHHGS after the N-terminal met (M+1 calculated 29637.1 Da). Fingerprint analysis of a proteolytic digest [trypsin (100 μg/ml) and chymotrypsin (100 μg/ml), in digestion buffer (Tris–HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM), KCl (50 mM)), 37 °C, 4 h, quenched with 0.05% TFA] of the protein (50 μg) by MALDI-MS (Micromass Tof-Spec 2E spectrometer using α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA as the matrix) showed peptide ions at m/z 816, 903, 1080, 1187, 1277, 1644 and 2167 a.u. corresponding to the predicted fragments—LAEQAER, VISSIEQK, YLAEVATGEK, DSTLIMQLLR, EHMQPTHPIR, NVTELNEPLSNEER, and TAFDDAIAELDTLNEDSYK, respectively. Circular dichroism in the 190–260 nm region was carried out using a Jasco spectropolarimeter with a protein concentration 20 μM in 10 mM phosphate buffer (pH 7.5). The spectrum was virtually identical to that of purified native 14-3-3γ from sheep brain (data not shown). Analysis using the DICHROWEB [18] program suggested a content of 70% α-helix and 4% β-sheet, similar to results obtained for other isoforms [19].

2.2. In vitro selection of aptamers

A degenerate oligodeoxynucleotide library was synthesized by phosphoramidite chemistry on a 1 μmol scale using an ABI Expedite™ 8909 Nucleic Acid Synthesis System, purified by HPLC, lyophilized and dissolved in 10 mM Tris (pH 8) at a concentration of 25 pmol/μl. The library, 5′-CCGAAGCTTAATACGACTCACTATAGG GATCCGCCTGATTAGCGATACT-[40N]-ACTTGAG CAAAATCACCTGCAGGGG-3′, was composed of 40 random deoxynucleotides flanked by primer annealing regions containing BamHI and PstI sites. The primers used for SELEX were BBBCCCCTGCAGGTGATTTTGCTC AAGC-3′ (B = Biotin-CE) and 5′-AGGGATCCGC CTGATTAGCGATACT-3′: the latter of these and 5′-C CCCTGCAGGTGATTTTGCTCAAGC-3′ were used for final cloning and sequencing.

For the initial round of selection the library sequences were 5′ labeled using γ-^{32}P-ATP: a sample of the aptamer library (20 μl, 500 pmol) was treated with γ-^{32}P-ATP (30 μCi, 3 μl), 20 units T4 DNA ligase (2 μl, New England Biolabs), T4 ligase buffer (10×, 5 μl, New England Biolabs), and water (19 μl) at 37 °C for 15 min. ATP (100 mM, 1 μl) was added, the mixture incubated for a further 45 min at 37 °C and separated on a 12% acrylamide/7 M urea gel. The labeled oligodeoxynucleotide band was visualised by autoradiography, excised and incubated in elution buffer (0.4 ml, 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8.0, 1.0% SDS) at 37 °C for 1 h. The solution was centrifuged at (13 K, 1 min, 4 °C) the supernatant removed, EtOH (0.9 ml) was added to the pellet and then gently agitated at 4 °C for 30 min before centrifugation (13 K, 30 min, 4 °C) and removal of the supernatant. The pellet was resuspended (200 μl 10 mM Tris–Cl pH 7.6, 25 μl 3 M Na acetate pH 5.2, 450 μl EtOH), incubated for 30 min at 4 °C then centrifuged (13 K, 30 min, 4 °C). The final pellet was rinsed with 70% aqEtOH (100 μl) and dissolved in TE buffer (60 μl, 10 mM Tris–Cl, 1 mM EDTA, pH 8.0).

14-3-3γ Protein stock solution (8 μl, 0.1 mg, 1.6 nmol) was added to a sample of the radiolabelled aptamer library (20 μl, 25 pmol) in sterile TE buffer (22 μl) and the solution agitated gently at 4 °C for 16 h. A slurry of Ni^{2+}-Sepharose beads (10 μl, protein capacity ~400 μg, Amersham Biosciences), prepared according to the manufacturers instructions, was washed with TE buffer (50 μl), added to the aptamer–protein solution and gently agitated for a further hour at 4 °C. The suspension was centrifuged (6.5 K, 2 min), the supernatant removed and 'SELEX Buffer A' (50 μl, 5 mM Tris–Cl, 150 mM NaCl, pH 7.5) added to the beads. These were agitated for 5 min and separated

by centrifugation (6.5 K, 2 min). The washing procedure was repeated a further three times. The beads were suspended in 'SELEX Buffer B' (50 μl, 50 mM Tris–Cl, 1 M NaCl, pH 7.5) and the washing procedure was repeated a further four times. The beads were suspended in 'SELEX Buffer C' (50 μl, 50 mM Tris–Cl, 6 M guanidinium Cl, pH 7.5) and agitated for 1 h at 37 °C. This was repeated with a further aliquot of 'SELEX Buffer C' and the supernatants from both final washes were pooled, diluted with water (500 μl) and desalted using a Supelco supelclean LC-18 SPE column (3 ml). The columns were washed with water (10 ml) and the ssDNA was eluted with CH_3CN:CH_2Cl_2 (0.6 ml, 3:1), evaporated to dryness and dissolved in sterile TE buffer (40 μl).

Amplification of ssDNA was carried out by PCR using a PE 480 Thermal Cycler (Perkin Elmer): Typically each reaction contained two Ready-to-Go™ Taq Polymerase beads (Amersham Biosciences), the aptamer solution (20 μl), the SELEX primers (12 μl, 500 pmol), 5′[α-^{32}P]dCTP (1 μl, 10 μCi), and water (15 μl). The mixture was heated at 95 °C for 5 min then subjected to 12–18 PCR cycles (95 °C–30 s, 54 °C–20 s, 72 °C–60 s). The number of PCR cycles was reduced to 12 after SELEX cycle 11 to reduce production of concatameric artifacts. The products were separated on a 1% agarose gel, extracted using the QiaQuick gel purification system and dissolved in TE buffer (60 μl). ssDNA aptamers (non-biotinylated strand) were separated from the complementary strand using streptavidin magnetic beads (New England Biolabs). Buffer equilibrated streptavidin magnetic beads (50 μl) were added to the PCR product (60 μl) and agitated gently for 1 h. The tubes were then applied to a magnet (Roche), the supernatant removed, and the beads were washed six times with NEB wash buffer (0.5 M NaCl, 20 mM Tris–HCl (pH 7.5), 1 mM EDTA) (0.5 ml), mixing by inversion. The ssDNA was removed from the beads by incubating in strand separation buffer (300 μl × 2, 50 mM Tris–Cl, 190 mM NaOH) for 15 min at room temperature, subjected to ethanol precipitation then resuspended in PBS-M (50 μl, PBS containing 1 mM Mg_2Cl_2, pH 7.4) and stored at 4 °C until the next round of SELEX. The amount of protein was reduced to 500 pmol (cycles 8–11) and to 250 pmol (cycles 11–16) in a binding volume of 50 μl. Counter selection was used to remove aptamers that bind Ni–NTA beads after rounds 3, 6, 9 and 12. A 50 μl aliquot of 10% slurry of Ni–NTA beads, pre-equilibrated into PBS-T, was added to the ssDNA in PBS-T and incubated with mild agitation for 20 min. The beads were discarded and those aptamers contained in the supernatant were collected and used in the next round of selection. Similar counter selection steps were taken in cycles 5 and 10 against His_6-tagged ferric binding protein C to remove aptamers that bind the hexahistidine sequence. Binding of aptamers was monitored by scintillation counting using a TRI-CARB 2100TR (Perkin-Elmer), and the UV absorbance (at 260 nm) of the bound and wash fractions. After rounds 11 and 16, the aptamers were amplified by PCR with the cloning primers, the products purified using the QiaQuick gel purification system, restricted with BamHI and PstI, ligated into pGEM® T-Easy (Promega) and used to generate *Escherichia coli* (DH5α) clones.

One hundred and eight colonies were picked from the round 16 cloning, the plasmids purified using the Charge-Switch® NoSpin Plasmid Micro Kit (Invitrogen) and the inserts sequenced on a PCR Sprint Thermal Cycler (Thermo Electron Corporation). Each sequencing reaction typically contained DNA template (5 pmol, 4 μl), primer (3.14 pmol, 1 μl), BigDye Version 4.1 (2 μl; PE Biosystems, UK) and water (2 μl). The reaction was cycled thirty times at 95 °C for 30 s, 45 °C for 15 s and 60 °C for 4 min. DNA sequencing was performed on an ABI prism 377 DNA sequencer. Sequence data was analysed using Contig-Express within the Vector NTI Advance™ V9 software package (InforMax). Several of the candidate aptamers sequences were synthesized using 5′-biotinyl CE-phosphoramidite as an affinity tag.

2.3. Aptamer-enzyme linked (ELONA) assay

A 96 well Immulon-4HBX plate (Thermo Electron Corporation) was coated with sc629 antibody (Santa-Cruz Biotechnology, SC-629), [100 μl in 10 ml coating buffer (PBS, pH 7.2, 14 mM NaCl, 270 μM KCl, 150 μM KH_2PO_4, 810 μM Na_2HPO_4)] for 1 h at 37 °C and stored at 4 °C overnight. After washing with PBS-T (2.5 mM $Na_2H_2PO_4$, 7.5 mM Na_2HPO_4, 0.5 M NaCl, 0.05% v/v Tween-20, pH 7.5), the plate was blocked by the addition of sucrose (2% w/v) in coating buffer (1 h, 4 °C) and subjected to a further washing step. 100 μl aliquots of the protein on test (typically 25 μg/ml in PBS-T) were added to each well and incubated at 37 °C for 1 h before a further wash step. The biotinylated aptamers (100 μM in PBS-T) were heated to 95 °C, cooled to room temperature and subjected to doubling dilutions in PBS-T. Aliquots (50 μl) of each aptamer solution were applied in triplicate to the wells and incubated for 2 h at 37 °C then subjected to a PBS-T washing step. Streptavidin horseradish peroxidase (Molecular Probes) was diluted 1:10,000 in PBS-T and 100 μl aliquots applied to each well. Plates were incubated for 30 min at 21 °C and washed again. 100 μl of TMB (Pierce) was added to each well and incubated at room temperature in the dark for 15 min. The reaction was quenched by addition of 100 μl 180 mM H_2SO_4 and the protein-bound aptamers–streptavidin complexes were quantified by determining the absorbance at 450 nm using a Wallac Victor II spectrophotometer.

2.4. Aptamer affinity purification of 14-3-3 from the CSF of a scrapie infected sheep

Two hundred microliters of CSF from a scrapie infected sheep (provided by the TSE Resource Centre, Institute for Animal Health, Compton) was added to 10 μl of the S2 biotinylated aptamer (10 μM). The aptamer had been

pre-heated to 95 °C for 2 min then cooled on ice for 5 min. The resultant solution was agitated gently for 1 h at room temperature. Magnetic streptavidin beads (10 μl) were added, the suspension gently agitated for 2 min and the unbound protein was washed from the immobilised aptamer. The immobilised fraction and the wash fractions were collected, denatured by boiling in Llaemmeli buffer, then resolved on a 4–12% Bis–Tris gel (Invitrogen), and transferred to PVDF for use in Western blotting using the sc629 anti 14-3-3 antibody.

3. Results and discussion

An in vitro ssDNA selection protocol was adopted in which His_6-tagged 14-3-3γ bound to Ni–NTA beads [20] was used to screen for high binding affinity aptamers from a library of up to 10^{13} different sequences [21]. Sequences which bound to the polymeric beads themselves or selectively to the His_6 tag were removed by counter-selection steps using unlabeled beads and beads loaded with His_6-tagged ferric binding protein C, a protein which shows less than 2% sequence homology with 14-3-3γ. The stringency of the selection was controlled by progressively adjusting the target protein concentrations, the incubation times and the washes. In order to assess our protocol, the enrichment of aptamers which bind 14-3-3γ was monitored after 11 and 16 rounds of selection. After eleven cycles multiple sequence alignments using ClustalX [22] of inserts sequenced revealed only modest enrichment of similar tetra- and penta-nucleotide motifs (3 and 4 of 14 sequences). The enrichment process was continued for a further five cycles, at which point the ssDNA retained at the protein-aptamer binding step leveled off at ~50%. However, sequence analysis of 94 clones generated at cycle 16 showed no consensus sequences. In fact, although there was a significant difference in the overall nucleotide ratios in the final set of sequences to that in the original library, phylogenic analysis [23] of the sequences indicated that these represented at least eighteen different aptamers families. The failure to obtain significant sequence convergence was frustrating but is not unique. Previous work on tubulin [24] and on HIV-1 SU glycoprotein [25] also generated groups of protein specific aptamers which share little sequence similarity.

To obtain some indication of which aptamers showed potential for use as 14-3-3 probes, twenty of the 40mer ssDNA sequences, chosen as representatives of the families identified by phylogenic analysis, were synthesised with a 5′-biotin label and screened for 14-3-3γ binding efficiency using an enzyme linked oligonucleotide assay (ELONA). Since our objective in this programme was the identification of a general, non isoform-specific 14-3-3 aptamer, the ELONA experiment was designed to eliminate those aptamers which bind to the N-terminal sequence of the γ isoform. Thus the sc629 antibody, which binds to the epitope, KSELVQKAKLAEQAERYDD (amino acids 4–22), was used to anchor the target protein to the plate. Two suitable biotinoylated aptamer sequences, S1 and S2, with K_D^{app}'s of 5.4×10^{-6} M and 5.6×10^{-7} M, respectively, were identified in this manner (Fig. 1).

These two compounds were screened against purified samples of six of the seven known 14-3-3 isoforms. The results (Fig. 2), which are corroborated by protein blot analysis (data not shown), show relatively small differences between the strength of binding of the biotinylated aptamers to the different proteins. No measurable binding to

Aptamer sequences		K_D^{app}(M)
BAGCGCCGGTGGTGGTGGGGGTTGTTGTTACGCGGTATTAT	S1	5.4×10^{-6}
BCGAGGCGTGTGCAAGGCGTGTTGTTCAAAGGTGTGTGTGT	S2	5.6×10^{-7}

Fig. 1. 14-3-3γ aptamers sequences, bars show areas of identity. B denotes biotin-CE. GTTG and GTGTGTG motifs were also present in several other aptamer sequences.

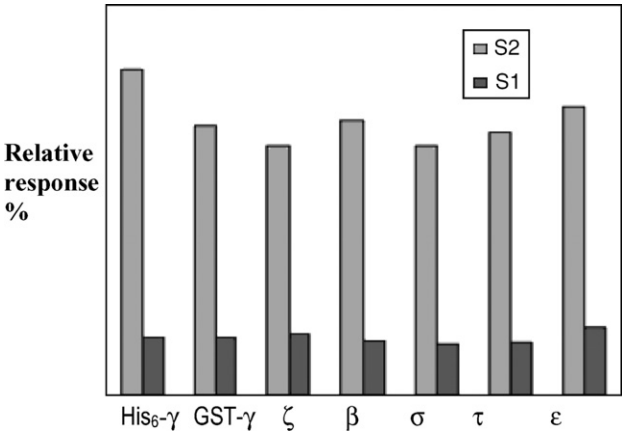

Fig. 2. Relative binding of biotinylated S1 and S2 aptamers to 14-3-3γ fusion proteins and 14-3-3 isoforms.

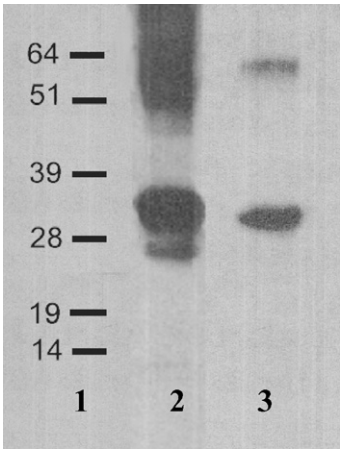

Fig. 3. Western blot analysis of the S2 aptamer affinity precipitate from a CSF sample from a scrapie infected sheep. 14-3-3 isoforms were detected using sc629 antibody. (1) molecular weights, (2) CSF, (3) 14-3-3γ standard. The identity of the high molecular weight material is under investigation.

control proteins (GST, BSA & ferric binding protein C) was observed. Two significant observations are evident—(a) that neither aptamer appears to associate with the proteins at the sc629 binding epitope, and (b) that in both cases the binding to all six isoforms tested is comparable.

The aptamers developed in this study are currently being evaluated as an affinity probes for 14-3-3 proteins in mammalian tissues and full biological studies will be published elsewhere. Here we give a single example of the use of the S2 aptamer for specific affinity precipitation of 14-3-3 from animal tissue. A sample of CSF from a scrapie infected sheep was incubated with the biotinylated aptamer. The aptamer bound protein was separated using magnetic streptavidin beads and the bound and unbound protein fractions analysed by SDS electrophoresis and Western blotting with the sc629 antibody. The affinity precipitated step led to significant enrichment of the 14-3-3 and enabled its detection at very low levels.

Fig. 3 shows the Western blot obtained in this experiment. It is noteworthy that the immunoprecipitation step did not require prior denaturation of the CSF sample since the aptamer binds effectively to the intact folded protein. This new method for affinity enrichment of the 14-3-3 isoforms from the CSF in neurodegenerative disease patients may lay the foundation for future development of robust diagnostic assays for TSE diseases.

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